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INDUSTRIAL MICROSCOPY

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INDUSTRIAL MICROSCOPY

A BOOK DEALING WITH
THE USE OF THE MICROSCOPE AND THE
PREPARATION OF SPECIMENS

FOR ALL WHO USE THE MICROSCOPE
IN INDUSTRY

BY

WALTER GARNER, M.Sc., F.R.M.S.

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PREFACE

The main object of this book is to present in one volume an adequate number of those methods of microscopic technique of general interest to the technical chemist. Further chapters describe the type of material likely to be encountered, without, of course, attempting a comprehensive treatment. The bibliography at the end of each chapter is intended to suggest the direction in which more detailed information may be obtained, and comprises the works which have been drawn upon in the writing of the following pages.

Chapters VI, VII, VIII, and XI had their origin in a lecture to the Society of Dyers and Colourists, and are reprinted from *The Industrial Chemist,* in which journal they appeared as a series of articles. I wish to thank the Editor of that Journal, and also Mr. S. Jackson in connection with part of Chapter XVI, for permission to make use of these articles.

Although most of the illustrations have been specially prepared for this book, there remains a number of figures of apparatus for permission to use which I have to thank Messrs. Baird & Tatlock, Ltd., Messrs. E. Leitz, Messrs. C. Baker, Ltd., Messrs. W. Watson & Sons, Ltd., Messrs. R. and J. Beck, Ltd., and The Cambridge Instrument Co., Ltd. Some of the remaining figures are taken from publications by The Macmillan Company, Messrs. P. Blakistons Son & Company, Messrs. Ilford, Ltd., and Messrs. Kodak, Ltd., and I am glad to acknowledge the kindness of these firms in giving me permission to make use of them. Further, Mr. G. Smith, M.Sc., and Mr. S. Jackson, have allowed me to reproduce certain microphotographs, whilst Miss M. Drucquer was good enough to draw three figures for me. As regards the text, The Williams and Wilkins Company, The Macmillan Company, and Messrs. Ballière, Tyndall & Cox, very generously gave me permission to quote freely from certain books published by them. Acknowledgments are made in all cases in the proper place in the text.

In the preparation of the manuscript, and in proof reading, I have been materially helped by suggestions and criticisms from Dr. Buckell, Miss E. Barber, Prof. J. B. Cohen, Mr. Ellis Clayton, F.I.C., Mr. V. D. Freedland, Miss I. M. Gilbank, B.Sc., Mr. F. Garner, Mr. Tompkins, B.Sc., A.R.C.Sc., and Mr. W. G. Watson, B.A. I am very pleased to take this opportunity of acknowledging my indebtedness.

W. GARNER

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INDUSTRIAL MICROSCOPY

CHAPTER I

INTRODUCTORY

THE choice of an instrument capable of fulfilling the many and varied requirements of the analyst is a very difficult matter under any circumstances. It is especially difficult for anyone who has had little previous experience of microscopic work to select from amongst the large number of available microscopes, differing widely in type, the one which will serve his purpose. In the following pages, the more important points which require consideration will be reviewed; their practical application is discussed in the next chapter.

The stand. It must be remembered that the microscope is not a complete piece of apparatus in the same sense as is a balance. The stand is the skeleton or framework upon which various attachments may be placed, in order to adapt it to the special work in hand. It is, therefore, very important to select a stand capable of being fitted with such accessories as occasion requires.

The weight of the stand is a point worth noting. It should be as light as is compatible with great rigidity, steadiness, and freedom from vibration. Unless it is required solely for photographic work, it should be sufficiently light to be conveniently moved about the laboratory.

The stand should be tested by turning the arm carrying the body tube and stage into the horizontal position, and observing if the microscope is still quite firm and stable. A stand not fulfilling this condition should be rejected.

Some makers construct the base as a tripod foot, whilst others prefer the horseshoe form. The horseshoe type is perhaps safer and more stable than the tripod variety, in addition to allowing adjustments to the substage to be made more easily. Many experienced microscopists, however, prefer the latter shape.

The body tube of the microscope is usually made with an internal diameter of 3 mm., but with microscopes intended for much photographic work the diameter is made considerably greater in order to minimize internal reflection from the sides of the tube. It is somewhat doubtful if much advantage is gained by this construction.

cM.

The body tube is fitted with a sliding draw tube, which carries the ocular, enabling the distance between the eyepiece and the objective to be varied. The position of the draw tube should not be too easily altered. Nevertheless it should not be so tight-fitting as to cause stiffness and jerkiness of working. If such is the case, it should be remedied by polishing, and not by the use of a lubricant. The draw tube must be graduated in mm. It should be borne in mind that the use of an objective possessing a longer setting than that for which the particular instrument is designed, or the employment of extra fittings, such as a triple nosepiece, causes the numbering to become inaccurate. For example, the addition of a triple nosepiece, measuring 15 mm., would cause

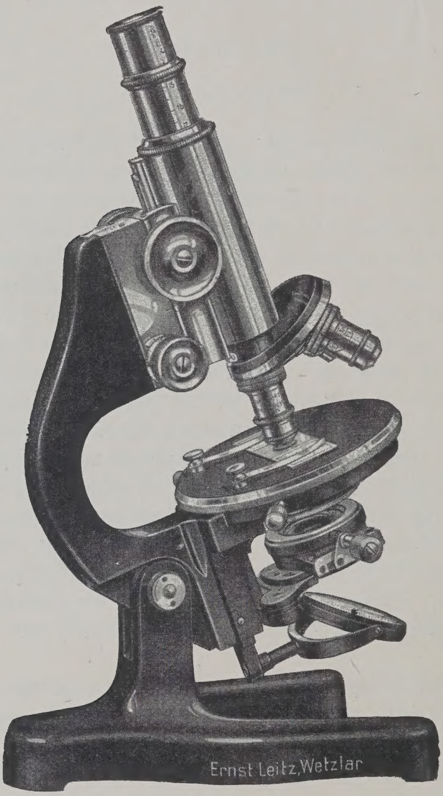

FIG. 1. THE LEITZ CT 23/83 MICROSCOPE

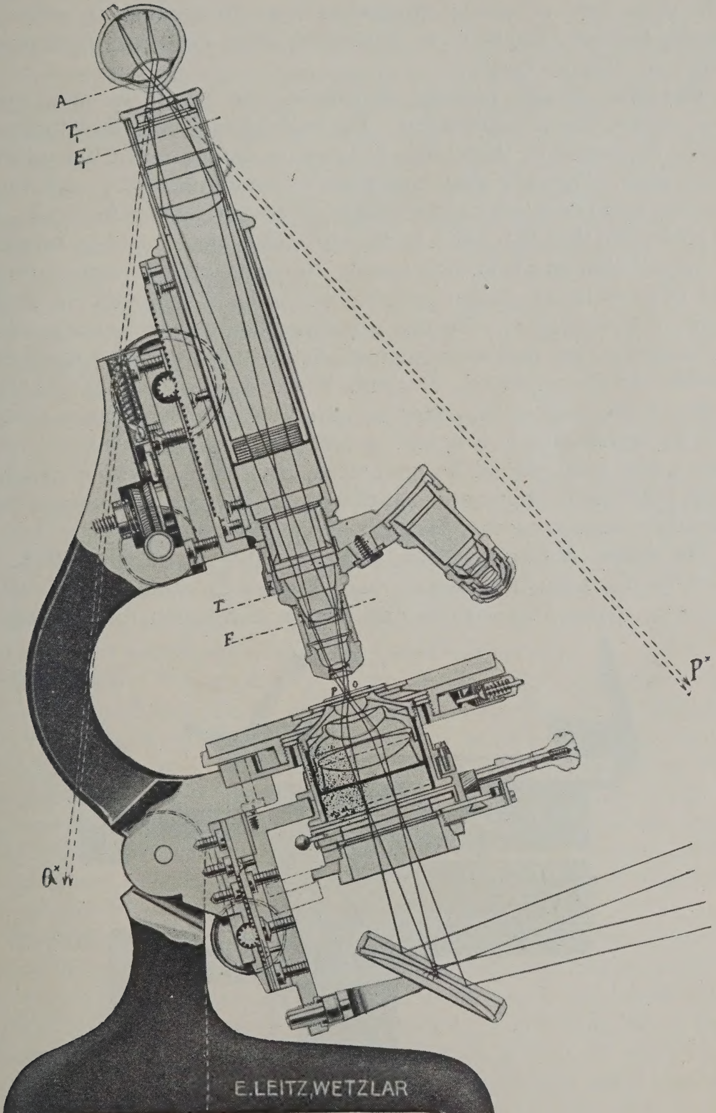
A
T_1
E_1
T
E
P^*
O^*
E. LEITZ, WETZLAR

FIG. 2. PATH OF LIGHT RAYS IN THE LEITZ CT 23/83 MICROSCOPE

the 160 mm. tube length to be indicated by 145 on the draw tube. The draw tube should be graduated down to 140 mm. in order to enable the tube length to be shortened, when using any such nosepiece or other accessory.

Focusing. Rough focusing is carried out by means of a rack and pinion coarse adjustment. The rack should be rather long, in order to permit of thick objects being examined under low power objectives. The rack work ought not to exhibit any play, and must possess some device for tightening so as to compensate for wear. It is preferable that the action be too stiff rather than too easy, because if the adjustment works very readily, the microscope is easily thrown out of focus by any slight pressure on the body tube (not the draw tube) or by vibration. The fine adjustment should not be too sudden in its action; the degree of fineness advisable is dependent upon the powers chiefly employed. If 4 mm. be the highest usual power, the value of five turns of the screw per mm. would be found satisfactory, but for higher powers it would be better to have ten turns per mm. The screw head should be graduated, particularly if photography with achromatic objectives is anticipated. The graduations are also useful for measuring the thickness of very thin objects.

The stage. A large circular stage, capable of being rotated, is *essential* for industrial work. Such a stage is always fitted with centring screws; these enable the centre of rotation to be brought

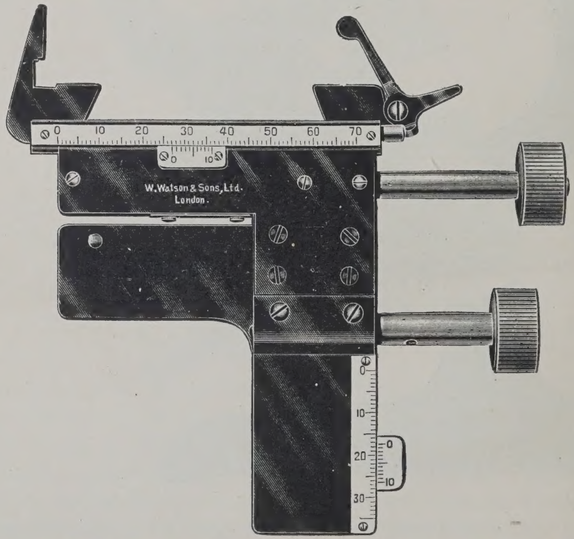

FIG. 3. THE ALPHA MECHANICAL DETACHABLE STAGE (*Watson*)

on to the optical axis of the instrument, causing the object to remain in the centre of the field when the stage is rotated. A mechanical stage is built into the structure of many microscopes, but for general purposes such a stage possesses many inconveniences. Detachable stages can readily be obtained, and may easily be fitted to a plain stage on the few occasions when a mechanical stage may be advantageous, i.e. when dealing with very small objects under high powers, or for systematic counting. The movement of such a stage should be sufficient to cover the whole of the width and two-thirds of the length of a 3 in. × 1 in. slide. The best forms of stages have vernier scales enabling the position of any object to be noted when the slide is in a known position on the stage and thus allow any particular feature to be readily located. The advantage of this will be realized by those whose work deals with the cross-sections of fibres, or with bacteria.

The substage. This must be capable of being swung out, and must have a screw-focusing arrangement. Some models are fitted with centring screws, which enable the optical axis of the condenser to be brought coincident with that of the objective. If the substage be correctly centred in the first place, and a triple nosepiece be not employed, the centring screws may perhaps be regarded as unnecessary for low-power work. They are essential, however, if the best results are to be obtained with medium and high powers, or with dark-ground illumination and high-power objectives.

The mirror. This should be plane on one side and concave on the other, and so mounted that its optical axis may be inclined in any direction at will. Its diameter should be at least 1·75 in. It is preferably supported by a metal rod built into the arm carrying the body tube and stage. If properly adjusted its optical axis will then intersect the optical axis of the instrument, and will coincide with this when the object is correctly illuminated. For photographic work, direct illumination is often employed, and it should therefore be possible to remove the mirror without difficulty.

Objectives. Familiarity with some of the more important theoretical points is necessary, as these have an essential bearing on the correct method of working. They will, therefore, be reviewed with special reference to practical requirements. Apart from these few fundamental details, however, not much benefit is to be gained by delving into the mysteries of compound lenses and their optical properties.

The Equivalent Focal Length. Usually expressed in millimetres, or on older English objectives, in fractions of an inch, this signifies that a simple convex lens having a principal focus lying at the distance mentioned, would produce an image of about the same size as the objective actually does. It is obtained from the formula—

$$\text{Magnifying power of objective} = \frac{160}{\text{E.F.L. in mm.}}$$

Working Distance. This is the distance between the object, when in correct focus, and the front lens of the objective. Modern objectives, of course, consist of a combination of lenses, and the higher the magnification given, the more lenses are required to produce a perfect image. The E.F.L. is usually measured from a point about half-way between the front and back lenses of the objective, and hence it always differs considerably from the working distance. For chemical and textile work, it is very important to choose objectives which possess long working distances.

Numerical Aperture. If the diameter of the lowest lens of the objective be taken as a base, and an isosceles triangle drawn to an object which is in correct focus, then the angle at the apex of the triangle is termed the *angular aperture* of the objective. It is obvious that this determines the amount of light which can enter the lens, and that the greater it is, the greater will be the area of the object which may be seen at one time. Abbé has shown that the brilliance of the image, which depends on this light-grasping power of the objective, is proportional to the sine of half the angular aperture. This value is known as the *numerical aperture*, usually condensed to N.A. It is extremely important as a guide to the properties of objectives, especially for high powers.

Resolving Power. This indicates the smallest distance which may separate two points, for them to be seen as distinct individuals under the microscope, with the objective in question. It is directly proportional to the N.A., and is usually expressed in terms of so many thousand lines per inch. The resolving power of an objective is far more important than its magnifying power. It is quite possible to construct a 4 mm. lens which will give a magnification four times as great as a 16 mm. objective though incapable of resolving such fine detail. The larger magnifying power would thus be "waste magnification." An indication of the resolving power of an objective may be gathered from its *optical index*, which is obtained by dividing the N.A. by the initial magnification, and multiplying the result by 1,000 to remove decimals. The higher the O.I. the better will be the performance of the lens in distinguishing fine details. A high O.I. also permits a higher powered eyepiece to be usefully employed. An objective must have an O.I. of 26 if all the fine detail is to be rendered visible by means of an x 10 ocular.

Depth of Focus. An objective possessing great depth of focus, or penetrating power, as it is sometimes termed, will bring into sharp definition a moderately thick object, such as a thick fibre. Depth of focus is inversely proportional to the N.A. and varies as the square of the E.F.L. It follows from this that objectives of high N.A. and usually, therefore, of high magnifying power, have a small depth of focus. In order to gain a clear idea of the structure of a comparatively thick object under high powers, the microscopist must depend largely on an imagined picture which he has synthesized from observations of the different planes successively brought into focus by means of the fine adjustment.

Correction of objectives. Most objectives are corrected for aberration by combining lenses of varying refractive index.

Aplanatic Objectives are corrected for spherical aberration which causes the outside of the field of view to be out of focus when the centre is sharply defined. Such objectives give a so-called "flat field," in which the definition at the edges of the field is almost as sharp as at the centre. Unfortunately even the best objectives can only be corrected to give a perfectly flat field at the expense of the definition obtainable at the centre. It is always found that fine detail is most clearly seen when the object is in the centre of the field. It should be added that the flatness of the field depends quite largely upon the eyepiece employed.

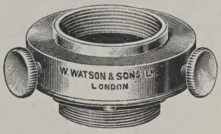

Fig. 4. Centring Nosepiece (*Watson*)

High-power objectives are usually corrected for use with a certain thickness of cover glass (known as No. 1) having an average thickness of $\frac{1}{200}$ in.; the tube length is taken as 160 mm.

When any other tube length is employed, the most obvious effect is to throw the image out of focus; if the length be increased, it will be found necessary to re-focus by moving the objective nearer to the object, which, of course, results in the working distance of the objective being shortened. The more expensive objectives have a collar which enables the distance between the front and back lenses of the objective to be altered; a simple trial will demonstrate that the working distance may also be shortened by lessening the distance between these two lenses, and refocusing.

In addition to the alteration of focus caused, there is a second result which is not very noticeable to the untrained eye. The effect on the re-focused image is the same as if the objective were under-corrected, for a certain amount of spherical aberration is produced.

When using low-power objectives for rough work, this distortion of the image caused by wrong tube length may be ignored, and, indeed, the tube length may be altered in order to obtain a rapid increase in magnification, with quite satisfactory results. With high-power objectives, however, this cannot be recommended, for the effect is much more marked.

The use of a cover glass whose thickness is different from that for which the objective is corrected, causes a more serious distortion. If the cover glass be thinner, the objective behaves as though it were over-corrected. The distance between the front and back lenses of the objective must then be decreased by means of the collar adjustment, if provided, or the tube length must be increased by means of the draw tube.

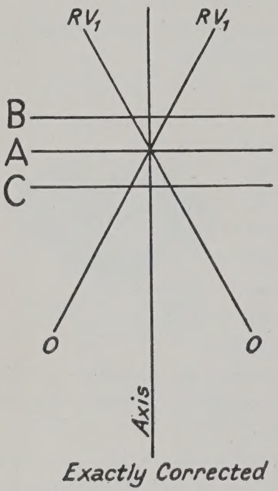

Fig. 5. Cover Glass Correct

The easiest way of demonstrating the effect is to illuminate some preparation by the dark-ground method. There will always be seen in the field some very small specks, which appear as brilliantly illuminated points, of no discernable shape, on the almost black background. One of these should be selected and focused exactly.

Fig. 5 illustrates the path of the light rays coming from the objective, assuming that the correct tube length and cover glass are being employed. All the rays come to a focus at *A*, and it will be seen that the illumination should be evenly distributed at two positions equidistant from *A*, i.e. at *B* and *C*.

The objective should now be moved towards the object by means of the fine adjustment. The point will expand into a small sharply-defined disc of light, which will not exhibit any noticeable difference of intensity over its surface. On moving the objective upwards, thus passing through the correct focus to a position where the speck is again out of focus, the appearance of the disc should be exactly the same as before. Thus the point appears the same on both sides of the correct focus.

Now, however, consider the case where the cover glass is too thin; all the rays from the objective will not come to a focus at the same point. Fig. 6 illustrates the path of the light rays in this case. The speck is approximately in focus at *A*. Upon moving the objective out of correct focus, towards the object, the point will still expand into a circular patch of light, but the disc will appear to have a bright edge and a dark centre, as is shown by the diagram at *V*. On raising the objective, through the position where the speck is in focus to a position where it is again out of focus (as at *R*), the disc will be seen to have a bright centre, and its edges will be misty, or foggy, and indefinite.

These effects may be corrected by increasing the tube length of the microscope. The practical method of adjusting the tube length according to the thickness of the cover glass is dealt with on page 20.

Achromatic Objectives. These objectives are largely corrected for chromatic aberration (which causes the image to have coloured fringes) by bringing two spectrum colours to focus in the same plane. In consequence, the chemical (ultra violet) and visible foci lie in different planes. Though this is not of any great importance in ordinary work, if a photograph be taken with one of these objectives in perfect visual focus, the resultant negative will be out of focus and blurred. The head of the fine adjustment requires turning a certain amount which must be found experimentally for each objective, in order to obtain the negative in correct photographic focus.

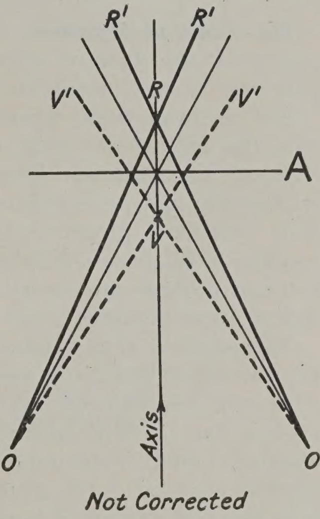

Fig. 6. Cover Glass Not Correct

To overcome this difficulty, and also to produce images which show no trace of chromatic aberration, *apochromatic objectives* were introduced, which bring three spectrum colours to focus in the same plane. The images are not congruent, and in consequence, coloured fringes are again obtained. This necessitates the use of special *compensation oculars* which are slightly over-compensated. The field obtained is usually not flat.

Formulae. The following formulae are of interest. It should be explained that λ for white light is taken to be 5,600, and that the constant 1·3 occurring in them is a factor, which is necessary because an objective is less efficient in practice than its stated angular aperture indicates.

$$\text{Available resolving power} = \frac{2\ \text{N.A.}}{1{\cdot}3\ \lambda}$$

$$\text{Optical index} = \frac{1000\ \text{N.A.}}{\text{E.F.L.}}$$

$$\text{Available illuminating power} = \frac{(\text{N.A.})^2}{1{\cdot}3\,\lambda}$$

$$\text{Initial magnification} = \frac{\cancel{250}}{\text{E.F.L.}} \quad 160$$

The choice of objectives. It will be evident from the preceding pages that the objective which will give the best result for one purpose may be very unsuitable for another.

For chemical work, where manipulations are often carried out with the slide on the stage and the object in focus, long working distances are essential. Many of the objects examined possess an appreciable thickness, and, hence, great depth of focus is also desirable. These two properties can only be given to an objective at the sacrifice of brightness of field and of resolving power. Fortunately, low and medium powers are employed for the most part, and the lack of these qualities is not a great disadvantage.

The majority of the higher-powered objectives are manufactured for the use of biologists, and the working distance is too short to meet the requirements of general analytical chemistry. If the analyst has to deal chiefly with botanical subjects, the working distance is often of less importance than the resolving power. Those workers engaged in the study of objects which present fine detail, requiring high powers, will use objectives possessing great powers of resolution and brightness of field, sacrificing depth of focus and working distance to obtain these.

When much photography is to be done, and only small areas in the centre of the field are to be photographed, apochromatics will probably be preferred, but if large areas are to be recorded special objectives and eyepieces should be obtained which give a flat field at the long distances between plate and objective which occur in practice.

The achromatic series is quite satisfactory for all but the most exacting work; their resolving power is adequate. They have a much longer working distance than the corresponding apochromatics, e.g. Beck's achromatic 16 mm. has a working distance of 6·25 mm., whilst the apochromatic only allows 3 mm.

The chief advantages possessed by the apochromatic objectives, apart from their convenience for photographic work, are that they give images without any trace of chromatic aberration, and have

ERRATA

Page 10, line three—

for $\frac{259}{\text{E.F.L.}}$ read $\frac{160}{\text{E.F.L.}}$

Page 50, twelve lines from end—

for $r = \frac{3M}{4}$ read $r = \sqrt[3]{\frac{3M}{4\pi\rho n}}$

where r is the average radius of the particles, ρ is the density, and M the total mass of the substance in the volume counted, and assuming . . . .

(5680)

usually a slightly higher N.A. than an achromatic objective of the same E.F.L. Although the difference in performance is not very great, apochromatics are undoubtedly to be preferred where very fine detail must be observed, or where slight differences in colour are to be distinguished. It should be remembered that the field is rarely flat.

The basic objectives required are three in number. The details given below refer to R. & J. Beck's achromatic lenses and illustrate the general properties of objectives of the powers mentioned.

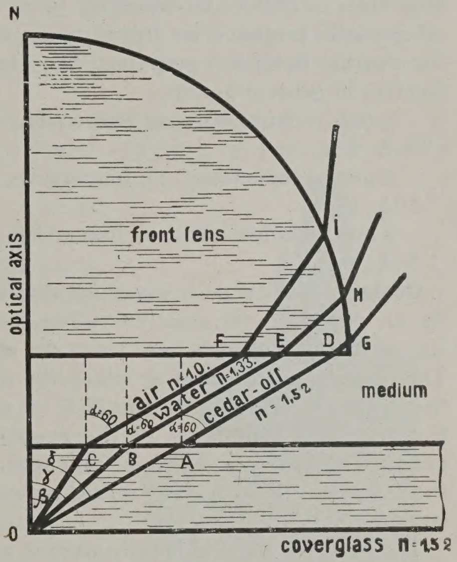

FIG. 7. THE INFLUENCE OF THE MOUNTING MEDIUM ON THE N.A. OBTAINED IN PRACTICE (*After Leitz*)

32 mm. Maximum field of view, 5 mm.; working distance, 22 mm.; N.A., 0·17; theoretical resolution, 15–18,000 lines per inch.

16 mm. Maximum field of view, 2 mm.; working distance, 6¼ mm.; N.A., 0·28; theoretical resolution, 25–30,000. This is an extremely useful power, and is most often used for the general examination of preparations.

4 mm. Maximum field of view, 1·2 mm.; working distance 1·5 mm.; N.A., 0·85; theoretical resolution, 81–88,000. This power of objective is excellent for examining in detail any structures which seem of special interest when seen under a 16 mm. It is almost the highest power which can be efficiently employed as a dry objective.

Three objectives of the powers given above will meet every requirement likely to arise in the course of general analytical work, excepting such as deals with bacteria. For this work objectives giving greater resolution are required.

High-power objectives. As a general rule, objectives with a focal length shorter than 4 mm. are designed as "immersion objectives," being so corrected that the best results are obtained when the space between the top surface of the condenser and the lower surface of the objective is optically homogeneous, and of the same refractive index as the glass of the objective.

The actual N.A. of an objective obtained in practice is decided by the lowest refractive index which is encountered by the light in its passage through this space, as may be seen by the diagram. The most customary method of obtaining optical homogeneity is by mounting the object in Canada balsam ($n = 1{\cdot}53$), and by interposing between the slide and condenser, and between cover-glass and objective, a little cedar-wood oil ($n = 1{\cdot}515$), which should be the oil specially prepared for this purpose, and not the cheaper oil used for clearing botanical preparations. Three types of high-power objectives in general use are—

3 mm. achromatic, oil immersion; initial magnification × 65, N.A. 1·2.

2 mm. achromatic, oil immersion; initial magnification × 90 N.A. 1·3.

2 mm. apochromatic, oil immersion; initial magnification × 90, N.A. 1·4.

Oculars. The eyepiece magnifies the real inverted image produced by an objective. It also determines to a considerable extent the size of the field of view, and has some effect on the flatness of field. The *eyepoint* is the point at which the rays come to a focus outside the eyepiece. The higher the N.A. of the objective, the greater is the area and brightness of the eyepoint and the greater is the magnifying power which may be obtained in comfort by means of the eyepiece. It is a great strain to work with eyepieces giving a large magnification.

The eyepiece most generally used is a ×8 or ×10. This may be supplemented by a ×5 or ×6, which gives a larger field of view, and a ×15 which gives a greater magnification.

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CHAPTER II

MICROSCOPE TECHNIQUE

The working of the microscope. In order that the most may be made of the expensive lenses and accurate fittings which are employed in modern microscopy, there has gradually evolved a somewhat elaborate procedure, which hedges round even such an apparently simple operation as the focusing of an object, with many precautions and adjustments.

These are much less formidable in practice than they appear on paper. It actually takes less time to adjust a microscope correctly (including the selection of the correct objective and ocular, the centring of the light, the adjustment of the tube length for cover glass thickness, etc.), than it does to read a description of the method.

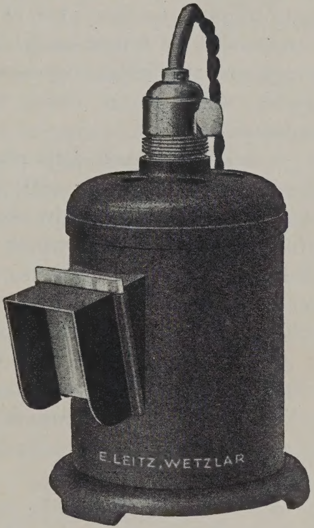


FIG. 8. THE LUCIFER MICROSCOPE LAMP (*Leitz*)

THE ILLUMINANT. Some of the best microscopists of the present day employ the old-fashioned paraffin lamp, and consider that this gives the best illumination for critical work. It will, however, be found that the most convenient and generally satisfactory illuminant for technical work is an opal electric globe.

The writer uses a 60-watt bulb, which gives an illuminated area of 2½ in. in diameter. This is situated at a height of 18 in. to 2 ft. above the surface of the bench, and 2 ft. away from the edge. A conical shield of narrow angle is arranged so as to shed a circle of light, about 3 ft. in diameter, on the bench, but long enough to ensure that no light enters the eye directly from the globe. The bench top is preferably covered with some black material, such as black leather or oilcloth.

When electricity is not available, gas is quite satisfactory. A sheet of ground-glass is placed in front of the mantle, or an opal globe is used, for the pattern of the mantle is otherwise in sharp focus when the condenser is correctly adjusted, and interferes with observation.

The ideal illuminant is an intense point of light, and the Pointolite lamp shown in Fig. 9 provides a sufficiently close approximation to this for practical purposes.

Naked lights which shine directly upon the bench should be avoided; the rest of the room is preferably in darkness, if any lengthy observations are to be carried out.

COLOUR SCREENS. These may be placed before the lamp, or in a special stop carrier below the condenser. They consist of pieces of coloured glass, or gelatine mounted between glass, or a solution in water of some dye, such as auramine, or salt, such as copper acetate.

Intelligently used, these screens have many advantages. Their most obvious employment is for tinting artificial light in order to gain a more correct idea of colour values of objects. A pale-blue screen will be found suitable for this purpose. They may also be employed to obtain greater contrasts. Bacteria stained faintly green, will become much more distinct if a pale-red screen be employed.

A third and most important use is based upon the fact that the resolution obtainable with an objective partly depends upon the wave-length of the light employed. It is generally accepted that the smallest particle which may be seen distinctly must have a diameter as large as half the wave-length of the light. Hence, using violet light, it should be possible to see a particle half the size of the smallest particle which would be resolved by red light.

Colour screens are very useful with dark ground illumination, objects of the same colour as the light disappearing from view.

The complete spectrum is somewhat as follows—

Rays	Wave-length
Röntgen . . .	0·1–1·0 milli-microns
Ultra violet . .	100–400 ,,
Visible . . .	400–800 ,,
Infra-red . .	800–1,000 ,,
Heat rays . .	1–10 microns
Short electrical .	1–10 mm.
Long electrical .	1 cm.–1,000 Km.

It is evident that even many of the bacteria are at the limit of observation with visible light; for example, some cocci are only 0·2 mu (or 200 milli mu) in diameter; they are thus perhaps 2,000 times the supposed diameter of the hydrogen molecule. Yellow light is mainly responsible for the resolution obtained with "white" light, and it would seem, therefore, that at least 30 per cent better resolution should be obtained when using an achromatic objective, if violet light be employed. Unfortunately this colour is very unpleasant and tiring, and in practice a yellow-green screen is found to be the most convenient. Nelson states that the ideal screen for visual microscopic work is one "which, filtering out the too pronounced red, softens down, but does not entirely cut out, the orange and yellow lights." He recommends the Wratten and Wainwright "Minus Red 4" screen, which gives excellent results. The Ilford Delta filter is also very suitable.

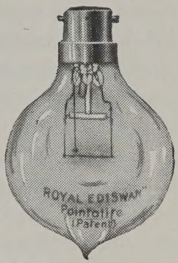


FIG. 9
POINTOLITE LAMP
(*Watson*)

Less increase in resolution is gained, of course, by the use of these screens with apochromatic objectives, than with achromatic.

For photographic purposes, a yellowish screen is normally required (Ilford Delta) if one must be employed. The screens, however, cut down the actinic light, and considerably lengthen the exposure.

It is occasionally necessary to construct one's own filters. An excellent and convenient method is to purchase a dozen of the lantern slides which are prepared for writing with pen and ink. If any difficulty is found in obtaining them, they may be prepared by coating one side of a clean negative glass with gum dammar in benzene.

The dry negative is simply immersed in a dilute solution of a suitable dyestuff until the film has stained sufficiently deeply. The required dyestuff may be found in Formanek's tables. (See also pages 31 and 71.)

GENERAL. The microscope should be placed near to the edge of the bench, and inclined at such an angle that the observer is in a quite comfortable position when his eye is applied to the eyepiece.

The unused eye should not be closed, nor should an eye shield be employed. It takes very little practice in order to keep both eyes open: the image presented to the brain by the unused eye then becomes unnoticed. The eyes do not become fatigued so soon by following this practice.

It is also a good plan to use the eyes alternately. Most people consider that they are able to see better with one eye than with the other. This eye should be employed mainly, resting it occasionally by using the other. In the writer's case, it is the right eye which is mostly used, the left eye only being used when making sketches or when the right eye becomes tired.

It is wise occasionally to close the eyes for half a minute or so. The eyes easily become tired, and this slight rest makes a big difference to one's powers of observation; also, if the mount is rather dimly illuminated owing to high magnification, the field seems to be brighter and easier to read than before. A dyer, when matching a shade, does not examine the patterns for any length of time, because he finds that this causes slight differences in shade to become less noticeable. It is exactly the same with the observation of detail. This explains, perhaps, why sometimes detail suddenly appears to be present, on looking at a mount with the left eye, which quite prolonged observation with the right eye had failed to note.

FOCUSING. After the slide has been placed in position on the stage, the objective should be lowered until it is closer to the slide than when in correct focus. With high powers, it is advisable to have the objective almost touching the cover glass. The field is now examined, the objective racked away slowly by means of the coarse adjustment, until it is roughly in focus, and finally focused by means of the fine adjustment. It may seem superfluous to mention that the microscopist must be quite sure which way he must turn the adjustments, in order to raise and lower the objective. It is very easy, indeed, for even an "old hand" to screw the objective down on to the slide. The least damage which can be caused is a cracked cover glass and the ruin of, perhaps, an important preparation, whilst the objective may be seriously injured.

The three objectives in a triple nosepiece will rarely be the same length, and this should be borne in mind when changing over from one to the other. It is wise to form the habit of raising the body tube by means of the coarse adjustment before turning the nosepiece. A longer objective, catching the cover glass, may spoil in a second a mount which has taken days to prepare. Objectives may be made *parfocal* by means of an adjustable collar, which can be set so that the higher powers are all in focus at the same setting of the focusing apparatus. They may also be *centred* by such a collar, so that the centre of the field of view is identical for all the objectives in the nosepiece.

Dust and dirt. On examining any object under the microscope there are always to be seen various specks of dust in the field; it is sometimes difficult to discover where these are situated unless a systematic test is made.

The slide should first be moved, and if the dirt is stationary, then it is obviously not in the mount. The eyepiece should now be rotated. In the majority of cases the dirt will rotate also, showing that it is somewhere on the ocular. The eye lens should be wiped with an old silk handkerchief, and if the dirt is still to be seen, it will be on the eyepiece micrometer, or on the lower lens.

Should the eyepiece prove to be quite clean, the condenser must be rotated slightly or thrown out of focus, and if the dirt moves, the condenser must be cleaned. If the dirt remains stationary, however, the objective is rotated a quarter of a turn, and if the dirt also rotates, it will probably be found on the back lens. This should be cleaned by means of a camel-hair brush. Quite a small amount of dirt in this position will seriously interfere with observation. If any dirt has become attached to the outside face of the lowest lens of the objective it must be removed by rubbing it with an old piece of silk moistened with xylene. Alcohol must not be used under any circumstances.

If the dirt has not yet been tracked down, it will be found on either the mirror or the illuminant, and a slight movement of these will settle the question.

Magnification. Magnification is far less important than resolving power. There is no theoretical limit to the possible magnification, but the resolving power in practice limits the useful magnification to about 750–900 diameters. In general analytical work such high magnifications are very rarely required. A ×10 eyepiece, used in conjunction with a 16 mm. objective, gives a magnification of 100, and with a 4 mm. objective, a magnification of 400 diameters. These are all which one will usually require.

Beginners have always a craving for high magnification. It should be the invariable rule to employ the lowest objective and eyepiece which will give the information required.

A magnification of 200 may be obtained by means of a 4 mm. objective (initial magnification ×40) and a ×5 eyepiece, or by means of an 8 mm. objective (initial magnification ×20) and a ×10 eyepiece. The two images of the object, though of the same size, possess different characteristics.

With lower power objectives, a large area of the body may be seen at one time. Errors of appearance due to imperfect focusing, incorrect tube length, incorrect illumination, and imperfectly-corrected objectives, are less noticeable. The depth of focus is greater, the illumination is better, and the eye is not so severely strained.

Magnification must, of course, be obtained by means of the eyepiece when a low-power objective is employed. The disadvantage of high eyepiece magnification is that the eyepoint becomes very small, consequently the eye quickly becomes fatigued, the image tends to become blurred and indistinct, and it is a great strain to work with a high eyepiece for any length of time. It must, however, be emphasized that a much truer idea of an object is obtained by this method of magnification than by using high-power objectives. Dark ground illumination should, of course, be conducted with a low power and a high eyepiece.

High-power objectives may be used for those flat preparations which do not possess any very pronounced structure, or which show a great deal of fine detail. Serious misconceptions are easily obtained if these high powers are unnecessarily used for thick, rounded, or angular objects, which have a distinct variation in thickness.

Corrections and adjustments. The following paragraphs contain a concise account of the procedure which is to be followed in adjusting the microscope for the examination of any object.

Each object presents a special problem in illumination, and the best results in any particular instance can be obtained only by experiment.

The adjustments are much more important in high-power work than with long focus and low-power objectives.

THE MIRROR. The direction of the light which enters the objective is determined primarily by the position of the mirror. The centre of the light beam should coincide with the optic axis of the microscope. When this condition is satisfied, it will be found that an accurately-focused image of the object will merely become blurred as a result of altering the focus by means of the fine adjustment. If the light is not central, however, but makes a small angle with the optic axis, the image will move slightly to one side, in addition to becoming indistinct, when the focus is altered. This may not seem of any special importance, but the false lighting does not give a perfect image, and with some objects may be positively misleading.

To set the mirror correctly, the iris diaphragm should be almost closed, and the mirror moved until the field is evenly illuminated. The light is then of necessity approximately central. The iris diaphragm should now be fully opened, and the mirror finally adjusted until the image remains stationary when thrown out of focus.

When a condenser is not employed the light may be easily rendered convergent, parallel, or divergent.

DIVERGENT LIGHT. This is obtained by placing the lamp a short distance away from the plane mirror.

PARALLEL LIGHT. This is best obtained by means of a bull's-eye lens. It is essential that the plane of the lens be at right angles to the imaginary line joining the centre of the illuminant to the centre of the mirror, for if incorrectly placed it spoils definition. The exact distance which must separate the bull's-eye from the illuminant, in order that the parallel light be obtained, is ascertained experimentally by focusing the lamp on a distant wall of the room, and should be recorded for future reference.

Convergent Lighting. Parallel light is employed, together with the concave mirror. This will suffice to fill the angular aperture of many lenses up to 16 mm., though for reasons of convenience a condenser is usually employed with 16 mm. objectives.

Oblique Lighting. This lighting may be obtained by swinging the mirror mounting until the centre of the mirror is an inch or so away from the optical axis of the instrument. In some microscopes the mirror arm is fixed, and oblique lighting must then be obtained by means of a stop with an eccentric opening, which is placed in the carrier under the substage condenser. A quick method of obtaining this lighting is by placing the tip of the second finger of the right hand against the lowest substage opening in such a way that light is not allowed to enter one-half of the condenser. By careful finger adjustment it is possible (with the lower powers) to obtain excellent dark ground effects by this unilateral illumination, which often supplies a new conception of the object under examination.

The substage condenser. The most widely-used form is known by the name of Abbé, who first popularized it. It is of simple structure, and although it concentrates the rays, it does not focus them exactly to one point. For very exact work there are achromatic condensers available which are carefully corrected to bring the rays to focus at one point. Whatever the type of condenser, the best results may only be obtained by centring, and the more carefully corrected the condenser is, the more accurately must it be centred. A very convenient method of effecting this is to close the iris diaphragm to a pin-hole, and raise the substage until the opening is clearly in focus by the objective. The circle of light is then brought into the centre of the field of view, by means of the centring screws on the substage.

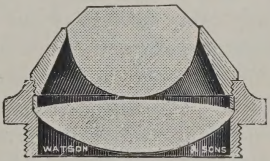


Fig. 10. The Optical System of the Abbé Condenser (*Watson*)

Before the condenser is working at its maximum efficiency, there are some further adjustments to be made. Some object must now be focused, and the condenser moved towards the stage until a clear image of the illuminant is also focused, apparently superimposed on the image of the object. The eyepiece is removed, and the condenser diaphragm closed until the back lens of the objective, seen down the microscope tube, shows a circle of light three-quarters its diameter. The eyepiece is now replaced and the condenser thrown very slightly out of focus. The illumination is now correct, and is termed "critical."

The best resolution is found in practice to be obtained when the condenser diaphragm is not open sufficiently to fill the angular aperture of the object glass completely. Excess of light causes "glare," which gives the object a misty appearance.

For many transparent objects, the iris opening will have to be closed even further than has been described, but generally speaking, the appearance is more likely to be accurate when using a wide beam of light than when only the central portion of condenser and objective are employed. When the condenser is correctly adjusted, the field will often be illuminated only in the centre, if a 16 mm. objective is in use. The whole of the lamp and fittings may be seen in the field, if the illuminant is some distance away. The condenser is therefore lowered until it is out of focus, when the field will be entirely illuminated. It is a simple matter to re-focus the illuminant by means of the condenser, just before changing over to the 4 mm. objective.

Condensers for high-power work. The Abbé condenser is badly corrected for both chromatic and spherical aberration, the result being that not all rays of the same wave-length, and not all parts of any one wave-front of light from the illuminant (with parallel lighting) come to focus in the same plane. Although for low-power objectives this is not a serious handicap, even with a 4 mm. it begins to be decidedly noticeable, and for 3 mm. and 2 mm. objectives, especially of the immersion type, a more accurately corrected condenser is essential. The aplanatic condensers sold by the best makers are usually also sufficiently achromatic, and many of them are so built that the top lens may be screwed off, the remaining combination being then used as an Abbé condenser (which in effect it is) for the lower objectives, the advantage being that a longer focal length is thus obtained.

Cover glass correction by tube length. This correction is rarely applied by inexperienced microscopists. This may perhaps be because many published descriptions of the method seem involved, or perhaps because it is only when the eye has become somewhat trained, that the value of the correction is realized. Nelson states that to an experienced microscopist the spherical aberration of an image, which is caused by incorrect tube length, is as objectionable as a badly-focused image. This is perhaps an over-statement, but there is no doubt that the correction is a most important one for critical work. After a little practice, the tube length may be correctly adjusted in less than half a minute.

It is much easier to adjust the tube length, using dark ground illumination, than when using the common transmitted light. There will always be seen in the field some very small specks, which appear as brilliantly illuminated points of no discernible shape on the almost black background. One of these should be selected, and focused exactly. Upon throwing the image slightly out of focus, the point of light expands into a small disc, and the appearance of this disc varies according to the correction required.

For Dark-ground Illumination. If, on lowering the objective from the position of correct focus, the disc has a sharp edge and a dark centre, whilst on raising the objective from the position of correct focus the disc has a foggy edge and a bright centre, then the cover glass is too thin, or the tube length is too short and the latter must be increased, or the distance between the front and back lenses of the objective must be decreased by means of the collar adjustment, if provided.

If, on lowering the objective, the disc has a foggy edge and a dark centre, then the cover glass is too thick, or the tube length is too great, and the tube length must be decreased or the distance between the front and back lenses of the objective increased by means of the collar adjustment, if provided.

The correct tube length may easily be found by re-focusing the speck, and turning the fine adjustment until the disc with a dark centre is seen. The drawtube is now altered until the object is again in focus, and the tube length is once more tested by altering the focus with the fine adjustment, and observing the appearance of the discs. The disc with the foggy edge is the more characteristic and the more easily recognized. It is improbable that the length will be correct at the first attempt, but two adjustments, or three at the most, will usually be sufficient.

It is perhaps somewhat more difficult to make the correction when observing an object by means of ordinary transmitted light. In this case, a very small speck, showing dark against the bright background, is chosen.

For Transmitted Light. If on lowering the objective, a dark disc with a sharp edge and a light centre is seen, whilst on raising the objective a dark disc with foggy edges is seen, then the cover glass is too thick, or the tube length too great.

The adjustment is best made with the condenser diaphragm fully open, since then the full aperture of the objective is brought into play.

(The correction must be made carefully when using dark-ground illumination, because the full aperture of the objective is always employed.) It may be mentioned, in passing, that a really good objective will require the same tube length, whether the condenser diaphragm be half or fully open. A poor objective, however, may require the tube length to be altered by several millimetres, if the condenser diaphragm opening be altered considerably. Such a lens would, of course, have to have the tube length corrected separately for dark-ground illumination, and the usual transmitted lighting.

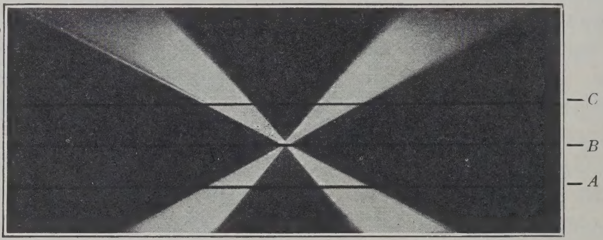


Fig. 11. Photograph of Light Paths in Dark-ground Illumination (*Leitz*)

A. Condenser too high; *B*. Condenser correct; *C*. Condenser too low.

Dark-ground illumination. The object is illuminated by means of light rays which leave the condenser at such an angle that they cannot enter the lens directly. Hence, the only light entering the objective is that which is reflected from the object, and the object therefore stands out brilliantly illuminated against a black background.

In order to obtain a perfectly dark ground, all the light rays which leave the condenser at such an angle that they would enter the objective, must be blocked out. This is done by placing an opaque stop in the centre of the condenser, as it is only the central rays which could do this. It follows that the N.A. of the condenser must be larger than that of the objective, and that the stop must cut out all rays of less N.A. than that of the objective—hence, the higher the power of

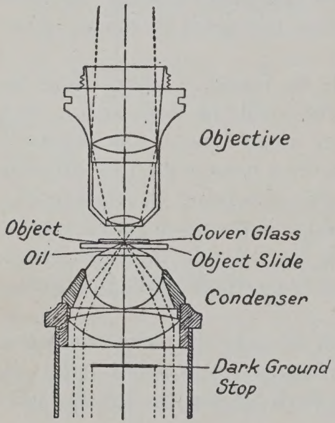


Fig. 12. Diagram of Dark-ground Illumination (*Leitz*)

the objective, the larger the stop which must be employed. It is also obvious that objectives with a high N.A. and short working distance should be avoided.

The ordinary Abbé condenser will give good results with objectives up to 16 mm. and fairly good with 8 mm., but poor results with a 4 mm. objective.

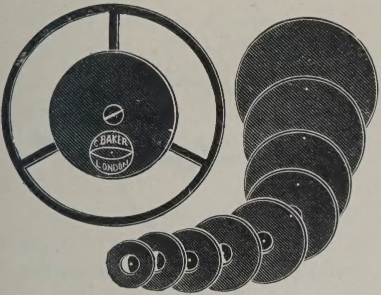


FIG. 13. DARK-GROUND PATCH STOPS (*Baker*)

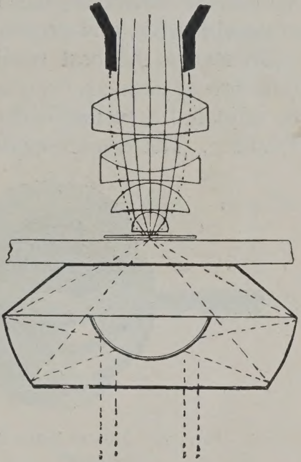


FIG. 14. BI-SPHERIC REFLECTING CONDENSER (*Leitz*)

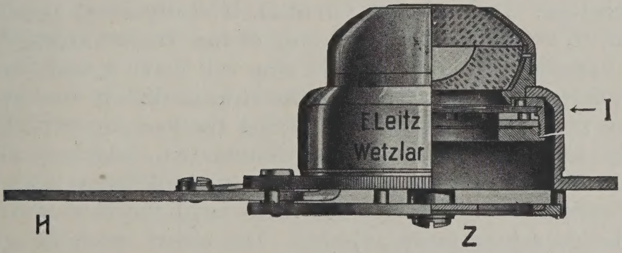


FIG. 15. REFLECTING CONDENSER HD 1·20 AA (*Leitz*)

The poor performance with higher powers is largely due to the fact that it is not sufficiently corrected to cut off the central light. An achromatic condenser will work quite well with a 4 mm. objective, with care. In order to obtain very accurately-focused light the reflecting type of condenser, such as the Zeiss Paraboloid, may be employed, but owing to the difficulty of grinding this type of surface accurately, perfect convergence is not obtained, with the result that glare is caused when large areas are to be illuminated. The Leitz bi-spheric condenser makes use of spherical surfaces only, which may be accurately ground, giving a dead sharp focus and a small illuminated area. Objectives may not have an N.A. greater than 0·65 for efficient use with a dry condenser. The usual N.A. for the special dark-ground condensers is, however, 0·9 and may be as great as 1·3. The Davis shutter, which is an iris diaphragm placed behind the objective, has the effect of lowering the working N.A. of an objective, and proves very useful in high-power work.

To secure the best results with dark-ground illumination, certain precautions are necessary. The mirror and condenser should be adjusted as usual, except that it is an advantage to use a ×5 eyepiece. The condenser diaphragm is now opened fully, and the patch stop inserted below the condenser in the place provided in the substage. It is almost essential, if really good results are desired, to use a variable patch stop of the Traviss type. A very slight variation in the size of the stop will make a very considerable difference to the result. If the stop is slightly too small, it will not prevent all light from entering the lens directly. If it is slightly too large it lessens the illumination, which is already meagre. The usual set of three patch stops of different sizes is not at all satisfactory. The stop is varied until the field is darkest, and the condenser focus adjusted. The object must be exactly at the crossing point of the light beams, or it will not be illuminated at all, and hence the position of the condenser is of the utmost importance. It will usually require to be moved nearer to the slide, from the position giving critical illumination.

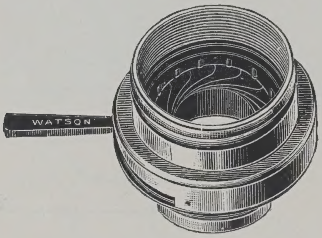


FIG. 16. DAVIS SHUTTER (*Watson*)

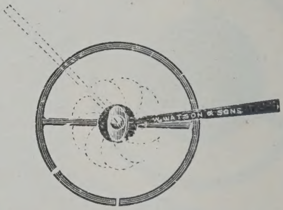


FIG. 17. TRAVISS EXPANDING PATCH STOP (*Watson*)

The tube length is now corrected and the low eyepiece exchanged for a higher one, when the results should be excellent.

The resolving power using dark-ground illumination is given by the expression $\frac{1}{4}$ N.A. objective $+\frac{1}{4}$N.A. condenser, and hence fine detail is difficult to obtain, but the general structure is often exhibited remarkably well. A large amount of extra detail and fine structure is often shown, but this is due to the greater contrast obtained. A transparent object, which may be almost invisible by transmitted light, will be thrown up in strong outline.

High-power immersion objectives. As already explained, immersion objectives used with cedar wood oil enable the full working N.A. of the objective and condenser to be made use of. The actual N.A. employed (assuming the possible N.A. to be 1·40) when the medium with the lowest refractive index encountered between condenser and objective is air, water, or cedar wood oil, is—

Dry mounted (air = 1·0) = cone of N.A. 1·0.
Oil immersion, water mounted = cone of N.A. 1·33.
Oil immersion, Canada balsam mounted = cone of N.A. 1·40.

The advantages of oil immersion are due to the obtaining of a concentrated cone of light; no loss by reflection, e.g. at cover-glass surfaces; and no loss by reflection at the objective of the wide angle rays (which are the most important in obtaining resolution).

In order to obtain these results the following practical points must be watched—

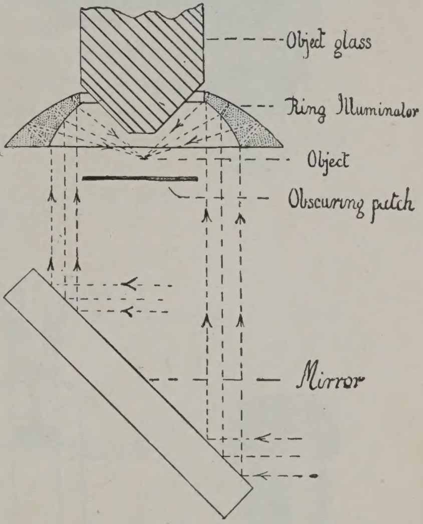


FIG. 18. ANNULAR ILLUMINATOR (*Beck*)

1. The area of the specimen illuminated must be no greater than the field of view, and preferably less, or "glare" will obscure detail. This is regulated by having a diaphragm stop at the light source.

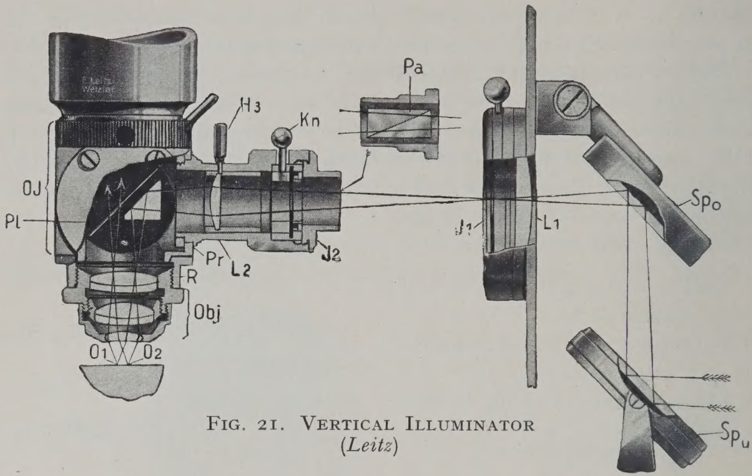
2. The condenser diaphragm should be adjusted almost to fill the aperture of the objective with light, i.e. on looking at the back lens of the objective with the eyepiece removed, two-thirds or a little less should be illuminated.

3. The object should be critically illuminated.

4. The substage condenser must be accurately centred.

Illumination of opaque objects. The main types of illuminants are—

1. The annular illuminator, in which light sent up past the object from the mirror is caught by an annular ring reflecting surface fitted round the objective rose, and reflected thence on to the object.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H3
Kn
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L2
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FIG. 21. VERTICAL ILLUMINATOR (*Leitz*)

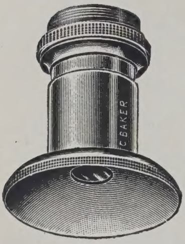

FIG. 19 LIEBERKUHN (*Baker*)

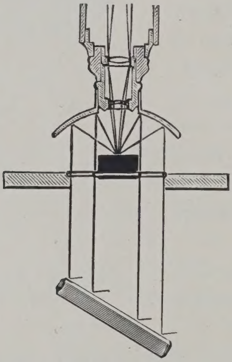

FIG. 20. DIAGRAM OF LIGHT RAYS IN LIEBERKUHN

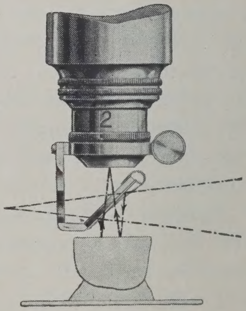

FIG. 22. SIMPLE VERTICAL ILLUMINATOR (*Leitz*)

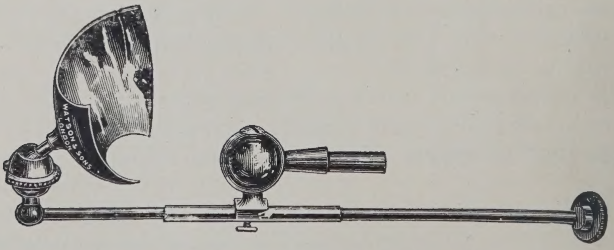
WATSON & SONS LONDON

FIG. 23. PARABOLIC SIDE REFLECTOR (*Watson*)

Fig. 18 shows Beck's aplanatic ring illuminator, and Figs. 19 and 20 the well-known Lieberkuhn.

2. The vertical illuminator, which is a reflector placed inside the microscope tube, reflecting light from a source at right angles to the optical axis of the instrument on to the object. Leitz and Watson both make a good model.

3. The side illuminator, which gives unilateral illumination, throwing the object up in strong relief. The model shown in Fig. 23 is typical of this construction.

The chief difficulty encountered when using a vertical illuminator is "glare," which may be caused by badly-centred illumination, too large a field illuminated, bright inside tube of microscope, dust on lenses or reflection from too convex back lenses of the objective (vertical illuminator only).

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CHAPTER III

ACCESSORIES

Spectroscopic eyepiece. It is very difficult to carry out useful work on any but the simplest of line spectra by means of the microspectroscope; absorption spectra only will therefore be considered.

The ordinary eyepiece is, for this purpose, replaced by a microspectroscopic apparatus, of which there is a number on the market; of these, however, only a few are suitable. They are usually built after the Sorby Browning pattern, which uses a compound direct vision prism to obtain the dispersion; two prisms of flint glass are placed alternately with three of crown glass, immediately above the eye lens of the ocular. The slit of the spectroscope is in the plane of the diaphragm. The most important feature of these instruments is the method of reading the spectrum. Although Formanek recommends a scale to be projected on to the spectrum (when an ordinary spectroscope is employed), this method is insufficiently accurate in microspectroscopic work, and it is essential to have a moving micrometer eyepiece which gives readings on an external vernier.

Other desirable features are a comparison prism, and a slit which, in addition to the normal adjustment for width of slit, has also some means of adjusting the length of the slit, in order that a small object, the image of which does not fill the full length of the slit, may be accommodated.

The calibration of the instrument follows the usual method; the vernier readings for the prominent lines of Na, K, Ca, Li, and Th, being plotted against wave-length, or better against log wave-length.

The study of coloured liquids requires a cell which enables either the thickness of the layer observed or the concentration of the solution to be varied; the former method is the better. The *Andrews cell* is very suitable. The outside container may be a small crystallizing dish; the dipping tube may be constructed from any wide piece of glass tubing, by grinding one end flat, and cementing to it a cover circle. By raising or lowering the dipping tube, the thickness of the observed layer may be varied at will. A low-power objective should be employed with such a cell.

When a few drops of liquid only are available, capillary tubes of various lengths may be used as containers. These are preferably though not necessarily, of an internal diameter greater than the diameter of the field of view of the objective. Externally, they should be covered with black paper, and the lower end should be coated with some opaque cement, such as Canada balsam mixed with lampblack. The tube may be supported vertically in a cork, which is fixed on to a slide by means of a little plasticine.

The absorption spectrum of a *transparent solid body,* such as a crystal or fibre, is determined by mounting it on a slide, and reducing the length of the spectroscope slit until the image projected by the objective is larger than the slit opening.

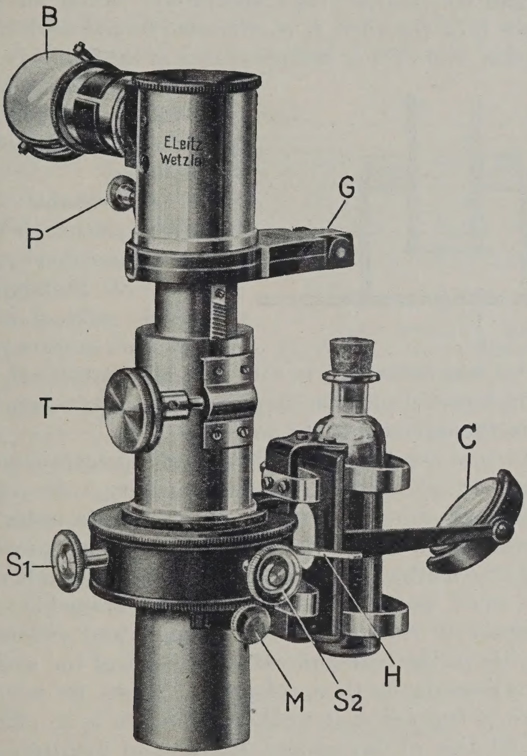

FIG. 24. SPECTROSCOPIC EYEPIECE (*Leitz*)

Opaque objects may be illuminated by means of any of the usual devices.

It is only rarely that a substance in solution exhibits only one absorption band, and still less frequently is the absorption symmetrical on each side of the line of maximum absorption.

In concentrated solutions the strongest band spreads out and swamps the smaller bands; it is therefore necessary to reduce the thickness of the solution layer carefully until all the bands are distinct.

The thickness is now further reduced until the weakest band is on the point of disappearing. Its position is taken, and the thickness decreased still further until the next strongest band is almost invisible, and its position read accurately. When all the bands present have been recorded, it is advisable to add acids and alkalis to the solution, any shift or disappearance of the bands being noted. The solution in alcohol, and also in amyl alcohol should be examined, if sufficient material is available.

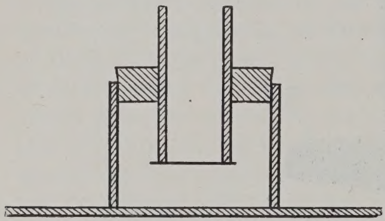

Fig. 25. Andrews Cell

It is advisable to use a buffered solution in water, the one recommended by the U.S.A. Bureau of Standards being 0·01 mol. each of acetic acid and sodium acetate per litre.

The initial concentration, in the case of commercial dyestuffs, for 1 cm. thickness of solution may be from 0·1 to 1·0 gm. per litre, according to the intensity of the colour.

Under the best conditions it is possible to identify 0·0001 gm. of dyestuff in 1 cc. of solution, by the capillary tube method. In practice, however, much experience is necessary in order to obtain exact identifications of the more difficult colouring-matters, but despite this, the method is more conclusive than any other available when only small quantities of material are obtainable. The most difficult colours to examine are the oranges and yellows, for the absorption lies in the blue and extreme violet of the spectrum. A good light is essential for these colours, or, indeed, for any colour; it is a mistake to suppose that weak illumination is an efficient substitute for dilution of the colour. Mixtures of dyestuffs present a more difficult problem, and not all mixtures give a satisfactory analysis.

Absorption of light may be *general*—over a large range of wavelength; or *selective*—over narrow spectrum bands. It is only in the latter case that the position of the bands can be determined, and the substance identified. The absorption may be either in the visible spectrum (e.g. a dyestuff) or in the ultra-violet (e.g. benzene), but special and costly apparatus, constructed with quartz lenses, is necessary for work in the ultra-violet.

At the present time no exact law has been formulated governing the relation between colour and constitution. There are, however, many rules and methods based upon the principle of analogy, which have found application in the determination of organic structure, and which are useful guides in analysis (see Cohen, *Advanced Organic Chemistry*, vol. ii, pages 70–146, and Bucherer, *Lehrbuch der Farbenchemie*, pages 156–290). Formanek's tables (*Untersuchung und Nachweis organischer Farbstoffe auf spektroskopischem Wege*) give the complete absorption spectra of a large number of dyestuffs, and the Colour Index of the Society of Dyers and Colourists also contains the summarized absorption spectra of most commercial dyestuffs.

White light is, of course, composed of a mixture of wave-lengths from 4,000–7,000 Å.U., which may be divided into three equal portions, blue-violet, green, and red, as shown in the diagram. A mixture of blue-violet and green lights gives the eye an impression of light blue, whilst a mixture of green and red lights gives the impression of yellow.

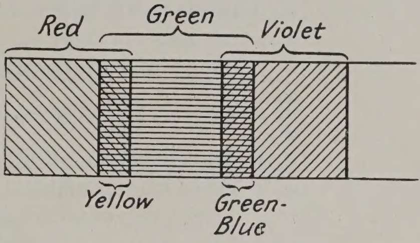

FIG. 26. DIAGRAMMATIC REPRESENTATION OF SPECTRUM

The colour of an object is due to certain wave-lengths being absorbed by that object, the resulting "colour" being thus produced by the residual wave-lengths of white light which have not been absorbed. The colours seen when light of various wave-lengths is absorbed is shown below.

Absorption	Residual Colour
Violet . . .	Lemon yellow
Blue . . .	Yellow
Green-blue . .	Orange
Green . . .	Magenta
Yellow-green . .	Purple
Orange . . .	Blue violet
Deep red . .	Sky blue

Bright clear colours are produced by a single narrow absorption band; dull-dirty colours are a result of a wide band or of more than one absorption band. A further factor in colour is the amount of "white" light transmitted, but this is outside the scope of the present discussion.

Typical absorption bands, one of which is followed by the majority of coloured substances, are shown in the diagram (after Formanek). The wave-length is plotted against the transmission density.

Two terms frequently encountered are "transmission density" (a) (Bunsen and Roscoe), and "extinction coefficient" (E) (Hurter and Driffield), which are defined by the following expressions—

$$I = I_o \cdot 10^{-ad} \ ; \ I = I_o \, a^{-dc} \ ; \ I = I_o \cdot 10^{-d a_o c} \ ; \ I = 10^{-ad} \ ; \ E = \log_{10} \frac{I_o}{I}$$

I_o = intensity of light entering solution.
I_o = intensity of light leaving solution.
d = length of path in cms. in solution.
$\frac{1}{a}$ = length of path reducing intensity to $\frac{1}{10}$.
c = concentration.
a_o = extinction coefficient for unit concentration and thickness of solution.

Polarizing Attachment. If a long string, held loosely between two points, is struck a sharp blow with a stick near one end, undulations are produced which occur in a definite plane, e.g. vertically. The string thus vibrating in a vertical plane could be passed through a grid with vertical slits without the vibrations being affected. Any attempt, however, to pass the string through a grid with horizontal slits would result in the vibrations being stopped.

Two strings may be set vibrating, one in a horizontal, the other in a vertical plane. A couple of experiments may be carried out by passing these two strings through (a) a horizontal, and (b) a vertical grid. The results which would be obtained are—

(a) *Slits vertical :* the vertical vibrations only would pass.

(b) *Slits horizontal :* the horizontal vibrations only would pass.

In each case, the total vibrations after passing the grid would be reduced by half, because the vibrations on one string are stopped.

An experiment could easily be devised to test the plane of vibration of the system of two strings after passing through a grid, by interposing in the path of the strings a second grid, which can be arranged with the slits vertical or horizontal at will. Assume now that the first grid employed was vertical; then on one string the remaining vibrations would be vertical, on the other nil, and the result, after passing the second grid (with slit orientation as stated below), would be—

(a) *Slits vertical :* the vertical vibrations would pass, and there would be, in this case, no change evident after passing through the grid.

(*b*) *Slits horizontal:* the vertical vibrations would be stopped and no vibrations whatever would pass.

This experiment with strings might be carried a little further by assuming a large number of parallel strings, vibrating in all planes round the path of motion of the strings. The first intercepting grid in this case would only allow vibrations to pass which were in a plane parallel to the lengthwise direction of its slits, and the grid could be rotated round the path of the strings without any difference being observed in the transmitted vibrations, in whatever plane the slits might be placed; although, of course, the transmitted vibrations would show a loss in total intensity, as compared with the initial total of vibrating strings.

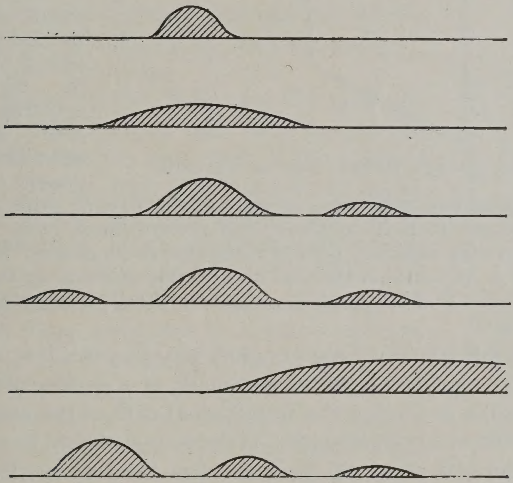

FIG. 27. TYPE CURVES OF ABSORPTION BANDS OF COLOURING MATTERS

In ordinary light the vibrations are assumed to take place in all directions at right angles to the ray, and the phenomena described with vibrating strings have a close parallel when plates of tourmaline are substituted for the two grids used in the experiments with strings. Tourmaline only allows light to pass through which is vibrating in a plane bearing a certain relation to its crystal axis, and this light so transmitted, which is termed *polarized,* will be transmitted or stopped by a second plate of tourmaline according as this is in the same position as the first, relative to the path of the incident light, or is rotated to a position 90° away from the first.

Tourmaline has certain practical disadvantages, the chief being that the light transmitted is coloured; it is therefore not widely used for the production of polarized light. The principal method of obtaining polarized light for microscopical purposes is by means of the Nicol prism.

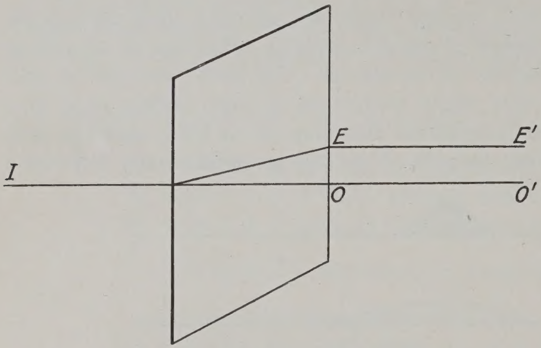

FIG. 28. DOUBLE REFRACTION IN CALCSPAR

Calcspar illustrates very clearly the property possessed by anisotropic materials, of splitting the incident unpolarized light into two component rays, in which the vibrations take place at right angles to each other, and which are termed respectively the ordinary and extraordinary rays.[1] The ordinary ray obeys the ordinary laws of refraction in its passage through the crystal, that is, the ratio of the sine of the angle of incidence to the sine of the angle of refraction is a constant, whatever the direction of the incident ray (Snell's law).

The extraordinary ray, however, does not obey this law, and in consequence, if the incident light falls normally on a surface of the crystal (Fig. 28), it does not follow the unrefracted path of the ordinary ray, but is refracted at a certain angle. If the source of light be sufficiently small, two images of it will be seen after the passage through the crystal—this phenomenon being known as double refraction.

The angular separation of these two rays inside the crystal is so small that it is difficult to mask off one whilst leaving the other

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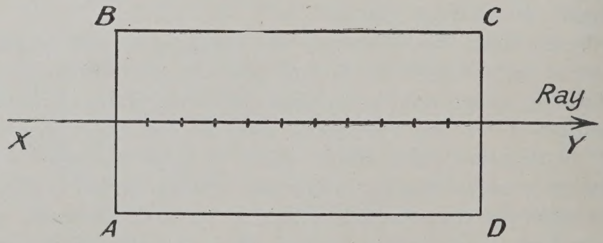
[1] The plane of polarization should not be confused with the plane of vibration. The diagram depicts the plane of polarization *ABCD* of the ray



XY, the plane of vibration being at right angles to this, the vibrations being indicated by the dots on *XY*.

(unless a very large crystal be used), in order to effect a separation. It may, however, be shown, by viewing the two rays through a tourmaline plate, that the planes of vibration of the two rays are at right angles to each other; in one position of the tourmaline the ordinary ray alone will be visible, whilst by rotating the tourmaline through 90° the extraordinary ray only may be seen.

Nicol Prism. Polarizer and analyser. A Nicol prism, which is usually employed to effect the separation of the two rays, is prepared by cutting a crystal of calcspar diagonally, and cementing the two halves together by means of Canada balsam, which has a refractive index of 1·55. The refractive indices in calcspar are, for the ordinary ray: $n=1{\cdot}658$, and for the extraordinary ray: $n = 1{\cdot}486$ (minimum).

Because the refractive index for the ordinary ray is greater in Iceland spar than in Canada balsam, total reflection will occur if the angle of incidence is greater than a certain critical angle, so that the ordinary ray will not be transmitted. The refractive index of the extraordinary ray in the spar being less than that in the balsam, it will be transmitted, and the light emerging will therefore be plane polarized in a plane at right angles to the principal plane of the crystal.

A second Nicol prism may obviously be used to ascertain the plane of vibration of the polarized light, in place of a tourmaline plate, and when employed in this way, it is known as an analyser.

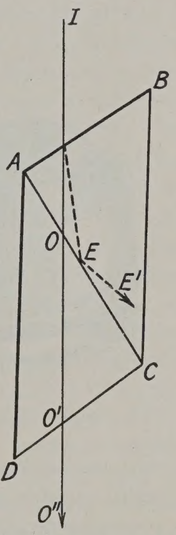


FIG. 29. PATH OF LIGHT IN A NICOL PRISM

AD, Canada balsam cementing layer; *IO″*, ordinary ray; *IE′*, extraordinary ray

When examining objects by polarized light under the microscope the polarizer may be placed in any part of the optical system before the light reaches the object, but it is usually fitted in the substage ring, in place of the Abbé condenser. When convergent polarized light is required, a small condenser may be fitted above the polarizer, though this is unnecessary, except for the observation of interference figures. Owing to the cost of these prisms it is usual to employ a small Nicol only, and because of this and the fact that more than half the light is lost during its passage through the polarizer, the concave side of the mirror should be employed, though this causes a certain amount of interference due to the light being slightly convergent.

The substage is provided with a notch into which fits a catch on the polarizer, so that it may be fixed in the same position every time it is used. The plane of vibration, which is indicated on the mounting, should be to and from the observer.

The analyser may also be placed anywhere in the optical system of the microscope after the light leaves the object, but the two most usual positions are immediately after the objective or over the eyepiece. The latter position is the more convenient, but is not suitable when high magnification oculars are employed. The mounting is fitted with a catch or stud which drops into a notch in the body tube of the microscope; it is to be noted that the body tube, which is usually capable of being rotated, should have a mark cut on it, and a corresponding mark on the body tube, so that it can be set in a definite position, or better still, by means of a catch, it should be prevented from rotating.

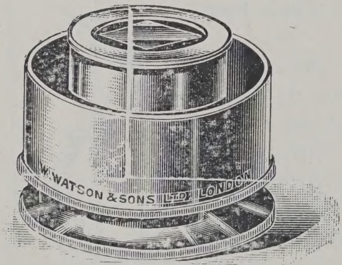


FIG. 30A. POLARIZER (*Watson*)

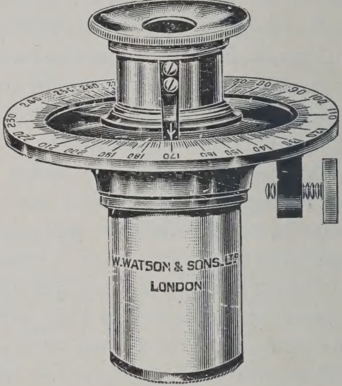


FIG. 30B. ANALYSER (*Watson*)

These notches do away with the necessity of adjusting the position of the components of the polarizing system, each time it is employed.

The analyser must rotate in its mounting, and must have a graduated circle indicating the angular rotation. The polarizer often rotates, but this is not so necessary.

The best forms of analyser have an eyepiece mounted under the Nicol, and in this case the cross-hairs of the eyepiece indicate the planes of polarization.

A suitable method of checking the correct setting of the polarizer and analyser is as follows: The rotating stage should first be exactly centred by means of the centring screws, after which a crystal of ammonium sulphate should be placed on a slide on the stage, and moved until it is in the centre of the field. The analyser is turned so as to be exactly crossed according to the setting, i.e. in the position of greatest darkness, and the stage is now rotated until the position of extinction, i.e. the position when the crystal is darkest, is obtained. One of the cross-hairs should now lie exactly parallel to the long edge of the crystal.

Optical anisotropy. An object which has no effect whatever upon polarized light, transmitting it with equal facility in whatever position it may be, is termed *optically isotropic.* Solid objects of this type are rather rare, and even these, if put into a condition of strain, become anisotropic, as may easily be shown by straining a glass thread under the polarizing microscope. The smallness of this class of substance makes the negative information of value.

The remaining substances behave similarly to calcspar in that they exhibit *double refraction* (*birefringence*), or *optical anisotropy.* The wave-front of light transmitted by them is ellipsoidal, and the index of refraction varies according to the plane of vibration of the incident light, or according to the direction taken by the light in the object under examination. An anisotropic body therefore resolves incident light into components which vibrate at right angles to each other; the planes of vibration of these bear a definite relationship to the crystalline form (using this term in a wide sense to include the structure of certain colloids). Thus, every anisotropic substance has *planes* (*or axes*) *of vibration,* one of which may be caused to coincide with the plane of vibration of the polarized light.

Examination with polarizer and analyser. The analyser should be set in the crossed position. An anisotropic crystal, such as ammonium sulphate or ammonium dihydrogen phosphate (almost all ammonium salts give strong polarization colours) is placed on a slide on the centred stage. When the stage is rotated, four positions will be found when the crystal is dark; and four positions, 45° away from these, when the crystal is bright; intermediate positions exhibit a gradual progression of luminosity. The crystal is said to exhibit *extinction* when it is in the position of greatest darkness.

Certain crystals, such as sodium chlorate, are *optically active,* and in addition to the above effect, rotate the plane of polarization; such a crystal will appear bright when the Nicols are exactly crossed; and can only be made to show extinction on the stage being rotated, by turning the analyser several degrees from the normal crossed position. This effect is only noticeable with thick crystals, and microscopically small crystals are too thin to give any trouble in this way.

Extinction is caused by the plane of vibration of the crystal being coincident with a plane of vibration of the crossed Nicols. To revert to the string analogy, the string vibrating in a certain plane finds a grid, represented by the crystal, with the slits in such a position that it can pass through without hindrance, but when the vibrating string reaches the second grid, i.e. the analyser, it finds the slits at right angles to its plane of vibration, and, in consequence, no vibrations pass through.

In the position of brightness (with the Nicols crossed), the planes of vibration of the crystal, instead of being coincident with those of the polarizing system, are at 45° to them. In this position, the polarized light coming from the polarizer, which is vibrating in one plane only, is, so to speak, again polarized by its passage through the crystal under examination, into two rays, the ordinary and extraordinary rays, vibrating in planes at right angles to each other. These two planes are neither of them crossed at right angles to the planes of vibration of the analyser, which, in consequence, transmits both rays partially, but it is important to note that during their passage through the analyser they are re-converted into light vibrating in one plane only, i.e. that of the analyser.

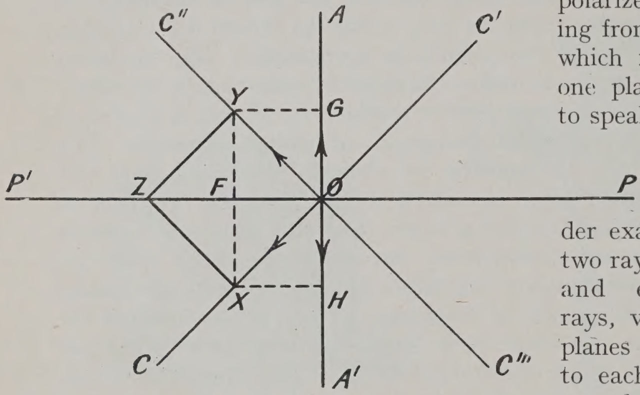


FIG. 31.

In Fig. 31, if PP' is the plane of vibration of the polarizer, AA' that of the analyser, and CC' and $C''C'''$ those of a crystal placed between the polarizer and analyser, then OZ is resolved during the passage of light through the crystal, in conformity with the parallelogram of displacements, into the two components OY and OX. The vibrations of these two rays are at right angles to each other, and hence, assuming that OY is retarded behind OX, interference cannot occur. During the passage of OY through the analyser, however, it is resolved into the two components OG and OF, the latter being absorbed, whilst OG passes through. Similarly, OX is resolved into OH and OF, the former only passing the analyser. As the planes of vibration of the two resolved components OG and OH are coincident, interference can now take place, having its origin in the retardation of OY during its passage through the crystal.

Retardation. An important effect is consequent upon this, however, for the light passes through the crystal in two directions, and these two rays do not travel at the same speed in the crystal, the slower ray being said to be *retarded* behind the other. This retardation depends upon several factors. *Firstly*, the refractive indices of the two rays decide their relative speeds, and when there is a considerable difference between these refractive indices, there is a correspondingly greater and strictly quantitative retardation of the slower and more refracted ray.

The difference between the two refractive indices, is a measure of the amount of double refraction exhibited by the crystal; when this difference is great, there is strong double refraction, when it is small there is weak double refraction.

Secondly, because the refractive index of the extraordinary ray varies with the orientation of the crystal with regard to the plane of vibration of the incident polarized light, its relative speed compared with the ordinary ray also varies. In consequence the observed retardation will vary according to the path taken by the light through the crystal, which is determined by the orientation of the crystal with regard to the plane of polarization.

The double refraction observed when the orientation of the crystal is such as to produce a maximum effect (i.e. when the refractive index of the extraordinary ray is at its lowest value), is a constant of the substance (of use in identification).

Finally, the thickness of the crystal (or more exactly the lengths of the paths of the two rays in the crystal) has an obvious influence, which is also quantitative, upon the amount of retardation.

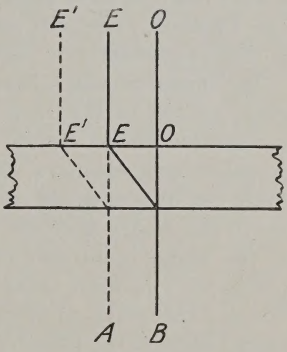


FIG. 32
DIAGRAM ILLUSTRATING RETARDATION

The effect of a crystal in producing retardation is not visible until the light has passed through the analyser, for although after leaving the crystal, one of the two rays has suffered retardation behind the other, and the two rays are therefore out of phase with each other, no interference can take place, because the planes of vibration of the two rays are at right angles to each other. Fig. 32 illustrates two rays, *A* and *B*, each of which is split up during its passage through the crystal; ray *A* gives an extraordinary ray *AE'E'*, and an ordinary ray *AEE*, the axis of which is coincident with that of the extraordinary ray from the light stream *B*, i.e. *BEE*. The extraordinary ray *BEE* and the ordinary ray *AEE* vibrate in planes at right angles to each other, and cannot therefore cause interference.

During the passage through the analyser, however, the two rays are brought into the same plane, and, hence, interference can, and does take place. For one set of conditions governing retardation, only one wave-length can exhibit this interference phenomenon, and can cause the resulting darkness on that wave-length; the remaining portions of the spectrum are unaffected. The total effect is that one of the colours composing the spectrum is subtracted, and, in consequence, the balance of spectrum colours which the eye recognizes as "white" is disturbed; the remaining unaffected portions of the spectrum together give the effect of colour.

The colour thus produced varies with the degree of retardation (caused by a combination of the three factors already mentioned), and the colour changes follow a definite order as the retardation becomes more and more pronounced. In effect, polarization colour is due to a narrow absorption band, which passes from one end of the spectrum to the other, with increase of retardation. These colour changes have been recorded for reference by several authors (see Chamot, page 281), and a chart of them may be obtained from E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany.

By means of such a chart, the retardation may be estimated with ease, except in the case of small retardations. In this case, the recognition of the degree of retardation is facilitated by means of so-called compensators, the most usual form of which is an accurately-cut quartz wedge, in which the faster component ray vibrates down the wedge from the thick to the thin portion. The thin edge of the wedge is introduced into the field in such a position that its planes of vibration make angles of 45° with those of the crossed Nicols; the plane of vibration of the slower component is then at right angles to the plane of vibration of the slow component of the specimen. By moving the quartz wedge so that its thickness at the optic axis of the microscope gradually increases, the retardation is correspondingly increased, and the polarization colours produced in the field follow the ordinary sequence.

As the polarization colours in the field increase in their order, due to the increasing thickness of the quartz, it will be noticed that the polarization colours originally exhibited by the specimen show a corresponding decrease in their order, because the retardation due to the quartz wedge is subtracted from the retardation due to the specimen. A point is eventually reached at which the substance is dark grey, whilst the field is the same colour as was the substance originally; the specimen is then said to be compensated. The compensator is marked with a scale which gives the order of the polarization colour at any point on it, and by reading off the scale the retardation of the specimen is obtained.

The orientation of the quartz wedge with regard to the specimen, in the position in which compensation is possible, indicates the plane of vibration of the slower component of the specimen, which is at right angles to the plane of vibration of the slower component of the wedge, i.e. roughly parallel with the long edge of the wedge.

Between the first and second orders of polarization colours, rapid change in colour occurs, from bluish-red, through blue, to yellow, and this colour change takes place with only a very slight increase in the degree of retardation. Use is made of this fact to gauge with much greater accuracy the retardation in cases where this is very slight, the specimen assuming merely a slightly-tinted dark grey appearance under the crossed Nicols.

This is done by the introduction of a *selenite plate,* which is of such a thickness as to produce alone the *first order red* colour. The slight retardation of the specimen, which alone gives a negligible polarization colour, when added to the retardation given by the selenite plate, is brought into play in that part of the scale of colours where slight alterations produce a considerable colour change, and its effect is much more readily observed. The field of view is, of course, red, due to the selenite plate, but the specimen becomes blue or yellow, according to the amount of retardation, when it is in the position of brightness with regard to the crossed Nicols.

The remarks with regard to the relative position of the plane of vibration of the quartz wedge and the specimen, apply equally to the selenite plate, which is merely a compensator with a fixed amount of retardation. When the planes of vibration of the slow components are at right angles to each other, the effects of retardation will be added; when they are coincident, the effects will be subtracted.

PLEOCHROISM. When an object is examined without the analyser, it in effect takes the place of the analyser, with the difference, of course, that the extraordinary ray is not stopped as in a Nicol prism. Therefore, in one position of the crystal, the ordinary ray is transmitted, whilst upon rotation through 90°, it is the extraordinary ray which passes through. The visible results obtained in this way are not striking, being in fact very little different from the appearance given by ordinary light, but this method of illumination occasionally throws more light upon structures than when the analyser is employed.

A striking example of this is obtained with so-called pleochroic substances. These bodies are usually coloured, and the majority of coloured substances behave in the way to be described. The absorption spectrum of the substance is different according as the ordinary or the extraordinary ray is examined, the result being that when the specimen is rotated, its colour is different in the two positions corresponding to the positions of extinction and of maximum brightness.

The effect is of considerable importance in the analysis of organic compounds, which are termed *dichroic* or *trichroic*, according as two or three distinct colours are seen on rotating the crystal.

When the light vibrating in a plane parallel to the longest side of the specimen is absorbed so as to give the darker colour, the specimen is said to exhibit *positive absorption.* When this light gives the paler colour, the absorption is negative.

Interference figures. (*Coniscopic observation.*) From the foregoing it is easily seen that if parallel light is replaced by convergent light (which may be obtained by the use of a substage condenser placed above the polarizer), the phenomena are complicated by the fact that the oblique rays have a longer path through the specimen than the rays incident normally, and, therefore, will exhibit a greater degree of retardation. The diagram illustrates that, with convergent lighting, the light which reaches the outer edges of the objective lens system has the longest path, and the greatest degree of retardation, whilst the light along the axis of the instrument has the shortest path and the least degree of retardation. This statement requires modification by consideration of the refractive properties of anisotropic materials, for the retardation depends also upon the direction in which the light passes through the specimen.

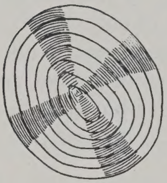


FIG. 33. INTERFERENCE FIGURE — ISOTROPIC BODY

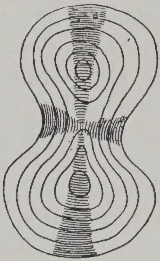


FIG. 34. INTERFERENCE FIGURE — ANISOTROPIC BODY

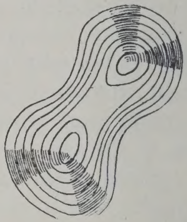


FIG. 35. INTERFERENCE FIGURE — ANISOTROPIC BODY WHICH IS ROTATED THROUGH 45° FROM FIG. 34

The effect may be examined by removing the eyepiece, and observing the back lens of the objective, though a better method is to employ a Bertrand lens, which is in essence a low-power microscope placed below the ocular, and which focusses the plane of the back lens of the objective. A further method, due to Klein, is to observe the interference figures at the eyepoint of the ocular, by means of a low-power magnifying glass, such as a focusing glass.

The effect observable depends upon the lighting employed. Monochromatic light exhibits the retardation due to interference, by producing darkness at those points, where the difference of phase is equal to one wave-length. These points lie on "contour lines" which vary in shape according to the optical properties of the specimen. Thus, an optically isotropic body would cause these contours to be more or less circular, whilst *uniaxial* doubly-refractive material would, in addition to these concentric contour rings, show a black cross reminiscent of the appearance of potato starch under crossed Nicols.

Biaxial anisotropic material exhibits two such crosses, or hyperbolic curves, and a figure eight arrangement of the contour rings, according to the orientation.

When white light is employed for observation, the above phenomena are complicated by the fact that the retardation is different for each spectrum colour, but the general principles are the same. The orientation of the optical axis of the material with respect to the optical axis of the microscope also plays a part in deciding the effect observed. (For further discussion of this subject see Chamot: *Handbook of Chemical Microscopy*, vol. 1, pages 288–308)

Crystal systems. The behaviour of crystals under polarized light is briefly summarized below, the process of examination consisting of first rotating the stage under crossed Nicols, observing the extinction position, and pleochroism, afterwards examining the interference figures produced with convergent lighting.

Between Crossed Nicols.

The field remains dark in all positions when the stage is rotated. The optical properties of the substance are then the same in all directions, and the substance belongs to the cubic system, or is amorphous.

Cubic System.

Crystal Axes Three, mutually perpendicular, equal length.
Refractive Index. One only, optically isotropic.

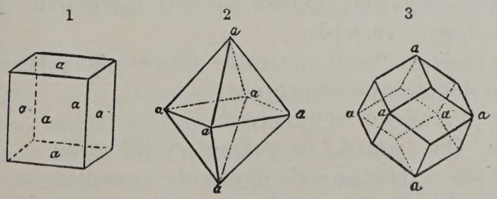


Fig. 36. Cubic System

Common Forms. Cube, octahedron, tetrahedron, etc.

Examples. Sodium chloride, sodium chlorate, alums, sodium uranyl acetate.

The crystal lights up when the stage is rotated. The substance is thus doubly refractive, and may belong to any of the remaining five crystal systems, which may, however, be divided into two groups according to their interference figures.

(a) *There is only one direction in which it does not light up.* The substance is then optically uniaxial, and the crystal system may be hexagonal or tetragonal.

TETRAGONAL.

Crystal Axes. Three, mutually perpendicular; two equal in length, the third variable.

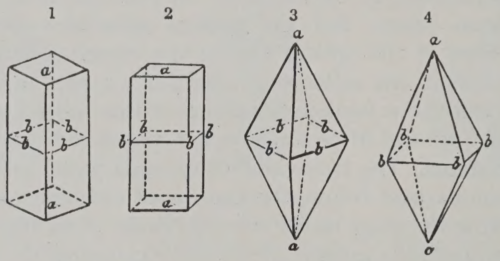


FIG. 37. TETRAGONAL SYSTEM

Refractive Indices. Two; optically anisotropic; may show pleochroism.

Extinction. Parallel or symmetrical.

Optic Axis. One, parallel to unique crystal axis.

Common Forms. Bipyramids, prisms.

Examples. p. brom phenol, urea.

HEXAGONAL

Crystal Axes. Four, of which three make an angle of 120° with each other, all being at an angle of 90° with the fourth. Three equal in length, the fourth variable.

Refractive Indices. Two; optically anisotropic (plates lying flat appear isotropic) may be pleochroic.

Extinction. Parallel or symmetrical.

Optic Axis. One, parallel to unique crystal axis.

Common Forms. Hexagonal pyramids, prisms, or plates; or rhombohedra with six rhombic faces.

Examples. Normal sodium phosphate ($12H_2O$) lead iodide, calcium carbonate, acetamide.

(b) *There are two directions in which the crystal does not light up.* The substance is optically biaxial, and the crystal system may be rhombic, monoclinic, or triclinic.

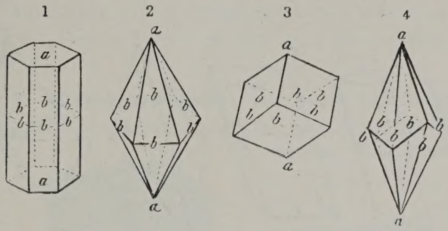


FIG. 38. HEXAGONAL SYSTEM

RHOMBIC SYSTEM.

Crystal Axes. Three, unlike, mutually perpendicular, lengths unequal.

Refractive Indices. Three, only two of which produce an observable effect in any one position of the crystal. Optically anisotropic. May show pleochroism.

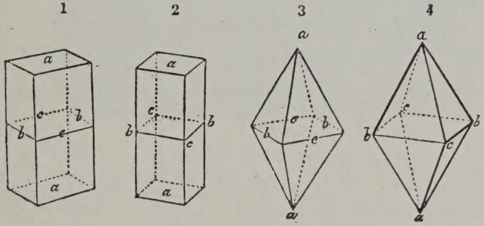


FIG. 39. RHOMBIC SYSTEM

Extinction. Parallel or symmetrical.

Optical Axes. Not parallel to crystal axes, rarely normal to crystal faces. Clear interference figures difficult to obtain.

Common Forms. Prisms, rhombs, rectangular prisms.

Examples. Potassium nitrate, silver sulphate, silver nitrate.

MONOCLINIC SYSTEM.

Crystal Axes. Three, two perpendicular to third, but not mutually perpendicular, length unequal.

Refractive Indices. Three, may show pleochroism.

Extinction. Parallel or symmetrical when light passes in plane of symmetry, otherwise oblique.

Optic Axes. Unrelated to crystal axes, or crystal faces. Clear interference figures difficult to obtain.

Common Forms. Prisms, flattened tablets.

Examples. Barium chloride ($2H_2O$), sodium sulphate ($10H_2O$), potassium chlorate, borax.

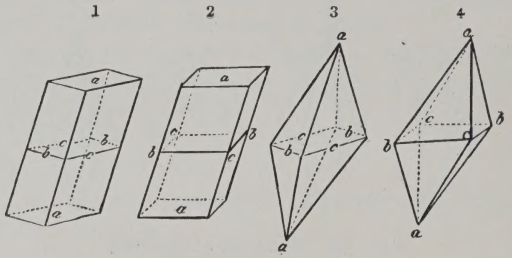


Fig. 40. Monoclinic System

Triclinic System

Crystal Axes. Three, mutually inclined, length unequal.

Refractive Indices. Three; may show pleochroism, which varies according to aspect of crystal.

Extinction. Oblique in all positions.

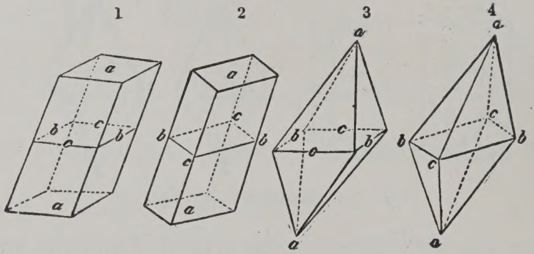


Fig. 41. Triclinic System

Optic Axes. Unrelated to crystal axes, or crystal faces. The interference figure depends on aspects of crystal.

Common Forms. Tablets.

Examples. Potassium bichromate, boric acid.

Polarization tube. For determining the rotation of optically-active substances, the Bates polarization tube may be employed. The objective of the microscope (and the condenser from above the polarizer) are removed The analyser is set in the cross position, and parallel lighting is obtained by means of an auxiliary condenser, such as a bulls-eye, in front of the light source Yellow, approximately monochromatic light should be used, by placing a suitable filter in the substage ring.

A known weight of substance is dissolved in a known number of c.c. of water, and the polarization tube is filled. The angle of rotation is found by taking readings on both the right and left of the zero point and averaging. By means of the formula, $\alpha_D = \frac{100a}{lc}$ where a is the angle found, c is the grammes per 100 c.c. of solution, and l is the length of solution examined, the specific rotation by sodium light is obtained.

Fluorescence in ultra-violet light. In recent times, quartz objectives have been introduced which enable the considerably increased resolution obtainable with the shorter wave-lengths of the ultra-violet region to be recorded photographically. This method must not be confused with the one now to be described.

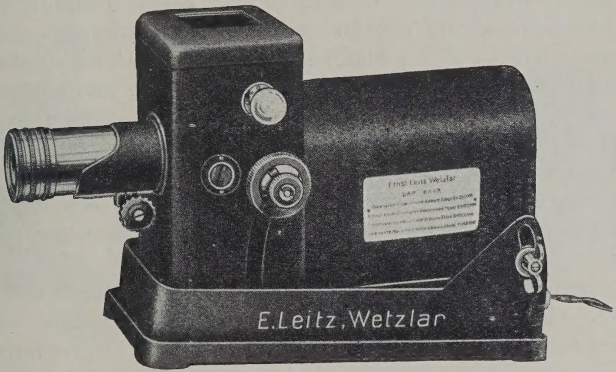


FIG. 42. LUMINESCENCE LAMP (*Leitz*)

When ultra-violet light falls on certain substances, it causes a fluorescence in the visible region of the spectrum, which may be observed under the microscope by means of ordinary objectives. There are, however, certain modifications necessary to the apparatus, due to the fact that ordinary glass cuts out wave-lengths below 3,100 A.U., thus causing the usual mirrors and condensers to be useless.

Furthermore, the light intensity available for illumination is ordinarily small, for from a good mercury vapour lamp, only about 20 per cent of the total energy is recovered as ultra-violet light after passage through a filter which blocks out the visible light. Not all this light, again, is effective, for only a portion of its energy is converted into fluorescence in the visible region.

The source of light may be a mercury vapour lamp, an arc lamp using iron cored carbons (Fig. 42), or a spark between the cadmium electrodes of a Leyden jar worked by an induction machine. The light is passed through a wood filter, which cuts out the visible spectrum but transmits the region between 2,000 and 4,000 Å.U.

When transmitted illumination is to be employed, the glass mirror must be replaced by either a quartz prism, or a silver reflector, and a quartz object slide must be employed. If a condenser is necessary, the ordinary Abbé form must be substituted by one made of quartz glass. The firm of Zeiss make these pieces of apparatus. When immersion objectives are being employed, cedar wood oil must be replaced by glycerine.

An ordinary objective is built up of glass which is slightly fluorescent; this decreases the contrast, and for this reason a special cover glass (also made by Zeiss) which is opaque to ultra-violet light, must be employed. This precaution is advisable from another point of view. A certain amount of U.V.L. is transmitted by the optical system of the microscope, and if allowed to enter the eye, would readily cause conjunctivitis; transmitted ultra-violet lighting should therefore never be used unless the eyes are protected by the use of such a cover glass, or by goggles of a glass which does not transmit the ultra-violet light.

The method of examination by transmitted lighting is difficult to employ. The simpler method employing reflected light has also its disadvantages, because it is difficult to obtain the required light intensity. A silver reflector may be employed, or for low powers which have a long working distance, the microscope may be placed close to the light source with the stage in such a position that it receives the maximum amount of light. The eyes should be protected.

The plane of the specimen may be found approximately by focussing a few small crystals of anthracene (or other highly flourescent body) which are placed on the slide; this simplifies the correct focussing considerably.

The method of analysis is still in its infancy, and there is an altogether inadequate amount of published information available.

Ultramicroscopy. The smallest particle which may be seen by the microscope with ordinary lighting is in the nature of 0·5 μ, but by means of the slit ultramicroscope particles much smaller than this may be detected, with a limit of about 10 milli μ, though they are not observed in the ordinary sense of the word.

Hydrogen molecule	0·1	milli μ.
Starch molecule	5·0	,,
Colloidal gold	10·0	,,
Microscopic visibility	200·0	,,
Average bacteria (diam.) . . .	1,000·0	,,
Naked eye particle size . . .	50,000·0	,,

The technique may be summed up by saying that a liquid containing the ultramicroscopic particles in suspension is illuminated from the side by a powerful beam of light, none of which enters the microscope objective directly, though a small portion of it is scattered by the particles which lie in its path. Much of this scattered light enters the microscope objective, thus indicating the presence of the particle.

When the particle is so large that it is capable of being resolved in the ordinary way by a microscope objective, the effect is similar to that obtained by dark-ground illumination, except that the illumination is from one side only. When the size of the particle is below the limit of microscopic resolution, it appears merely as a bright spot. This "image" is merely a diffraction pattern produced by the reflected light; consequently the resolving power of the objective ceases to be of primary importance, depth of focus being considerably more important, and the method of illumination of the utmost importance.

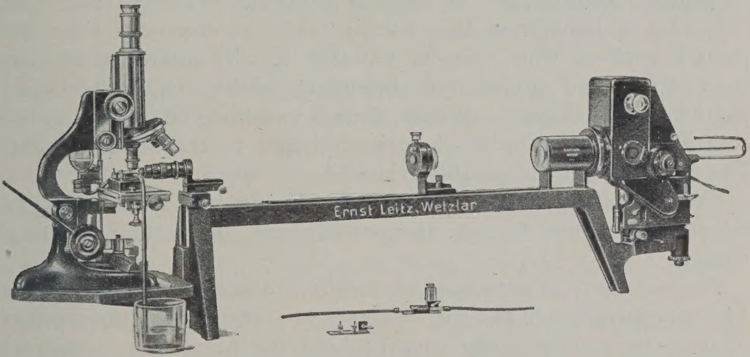


FIG. 43. SLIT ULTRAMICROSCOPE (*Leitz*)

The slit ultramicroscope of Leitz, based upon the original instrument of Siedentopf and Zsigsmondy, illustrates the features of a modern instrument.

The component parts are mounted on an optical bench, at one end of which is placed an arc light, the light from which passes through a collimator with a diaphragm which regulates the amount of light reaching the microscope. The light is focused on a precision slit, the width of which may be accurately controlled, and from this enters an illuminating objective which focuses the light on the optic axis of the observation microscope. The microscope incorporates special features, amongst which is a device for raising or lowering the stage, combined with a vernier gauge. The observation part of the microscope follows the usual lines. Quartz cells are provided to contain the colloidal suspension which is to be examined.

A method of illumination which is a development of dark-ground lighting, is used in the Cardioid ultramicroscope (Siedentopf), but it is considerably less useful than the slit instrument.

The Brownian movement is well known to occur when any particle sufficiently small in size (less than 5 μ) is suspended in some medium, such as air or water. The direction of movement is very erratic; its intensity is diminished by increase of viscosity of the suspending medium, and by increase in the size of the particle. The reflection of light from the particle takes its character from the particle surface; thus, metallic particles usually scatter more light than others, and appear more luminous, whilst irregularly-shaped particles, such as specks of fibre, show a twinkling effect, due to the rotation presenting sides of various shapes to the incident light. The reflected light is usually polarized.

The phenomena exhibited by a colloidal suspension or gel depend largely upon three factors, the particle size, shape, and electrical charge.

The *size* may be estimated by means of the slit ultramicroscope. The concentration of the suspension is so adjusted that the number of particles can be easily counted, and the number in a definite volume is then estimated. This volume is determined by measuring off in the micrometer eyepiece a definite length and breadth of the field, the depth being the thickness of the beam of light from the slit, in the liquid. It is necessary to make a number of counts, because the movement of the particles makes accurate counting very difficult.

Having obtained the average number n of the particles, the size may be calculated by means of the formula $r = \frac{3M}{4}$; where r is the average radius of the particles, assuming (which is hardly ever more than approximately true)—

1. That the density of the substance in its normal form is the same as when in the form of a dispersion.

2. That the total mass M of the substance in the volume counted, which is obtained by analysis of the suspension, represents the true mass of the substance (that is, for example, the substance has not become hydrated by the process of dispersion).

3. That the particles are spherical, and of uniform size.

4. That none of the material is in the true solution, or in the form of particles composed of very small molecular aggregates, too fine

to be detected by the method (with the slit ultramicroscope the practical limit is about 20 milli μ).

5. When a dispersion has been diluted, that no change in the size of the particles has taken place due to agglomeration, or other action.

The size of the particle has also a great influence upon the colour of the dispersion. Most substances, when the particle size is about 0·5 μ, cause the reflected light to be blue, the transmitted light taking on the complementary colour, orange.

Larger particles have little influence on the colour of the light; smaller particles often produce complicated phenomena according as they are transparent or not, and according to the viscosity of the suspending medium.

The *shape* of the particle may vary considerably, being approximately spherical (metals), entirely irregular (fibre particles), or crystalline (many chemicals). There is a very pronounced tendency for the micellae of organic materials which form gels, to assume a definite form, and also a definite disposition, which may be paralleled by the arrangement of molecules in crystals.

The *electrical charge* borne by particles is of the utmost importance, as it explains many of the phenomena which occur in the employment of colloids in industry. It may be determined by observing the cataphoresis ensuing when an E.M.F. is applied to the dispersion, so that a homogeneous fall in potential results along the cell containing the liquid.

Amongst the problems which may be investigated by means of the ultramicroscope are—

The interaction of colloids of opposite charge, in precipitating each other.

The effect of warmth, dilution, electrolytes, and peptizing agents upon the stability of emulsions such as rubber latex, and suspensions such as colloidal wax.

The structure and orientation of the micellae in gels, such as starch paste, and in solids, such as artificial silk.

Small accessories. Many of the instruments used in histological work are generally useful in microscopic analysis Such are: *forceps* with very fine tips, meeting accurately, and only slightly corrugated on the inside of the jaws; *needles*, which may be ordinary sewing needles, fixed in wooden penholders; *knives*, varying from a sharp penknife with small blades, to needles with the end shaped into a tiny scalpel; *scissors*, preferably with fine curved blades; *spatulas*, beaten out of a thick platinum wire, straight (screwdriver-shaped), and spoon-shaped, fixed in glass rods; *glass rods*, 10 to 20 cm. long, drawn out at the end to various degrees of fineness; *lengths of capillary tubing*, 10 to 20 cm. long, which serve as pipettes, and which may be calibrated if desired

Catalogues of apparatus employed by dentists and jewellers furnish many useful pieces of apparatus, such as small chisels, vices, clamps, etc. Other pieces of apparatus are described in their appropriate places in the chapters on general technique.

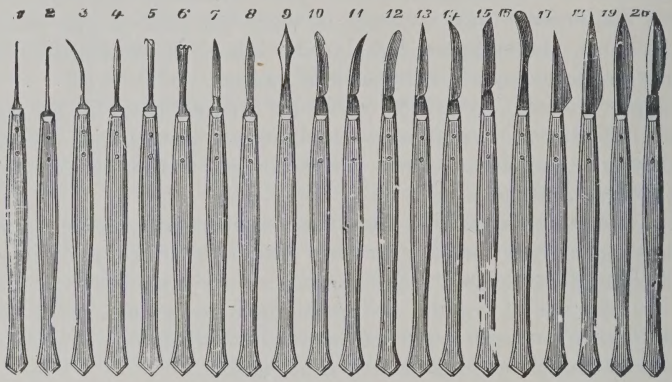


FIG. 44. SMALL ACCESSORIES (*Baird and Tatlock*)

Microscope slides are sold in several quantities, but the best are quite cheap, and it is wise to get them. They should be thin, free from flaws, and colourless. For chemical analysis, however, the cheap common greenish slides are preferable, as they are more resistant to the action of chemicals, particularly to alkalis.

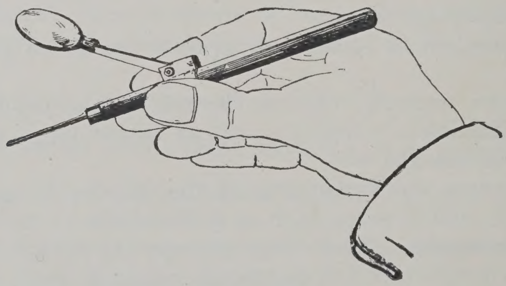


FIG. 45. HAND LENS (*Baird and Tatlock*)

Cover glasses are sold in three thicknesses, the medium thickness, known as No. 2, being suitable for general work; the thinnest cover glasses are essential for high-power work. The thickness of the cover glass is important when high powers are being employed, and may be measured by a special instrument in order that the objective may be adjusted, when it is provided with a collar for this purpose. As regards size, the $\frac{7}{8}$ in. cover circles will be found convenient, but for some purposes the square or rectangular form is preferable, an example being in the mounting of ribbon sections.

Surfacing of metals for examination. A cube of 1 cm. side is prepared, care being taken that the metal is kept cool during the cutting. The edges of the surfaces which are to be held in the fingers during the polishing should be slightly bevelled or rounded, because otherwise they may cause considerable pain when gripped tightly for a quarter of an hour or so, which is the usual duration of the surfacing. The following stages are to be carried out—

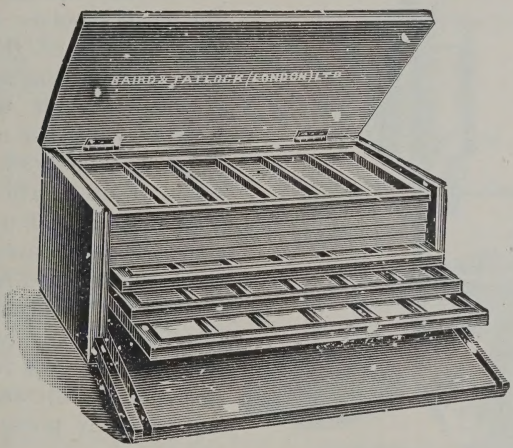


Fig. 46. Slide Cabinet (*Baird and Tatlock*)

Filing. The surface to be examined is filed flat on a rough single-cut file. The file is placed on the bench, and the specimen rubbed backwards and forwards on it. It is advisable to clean the teeth of the file between every stroke with a file "card" or brush. Great care must be taken in this and subsequent stages to avoid the surface becoming curved, due to a slight rocking movement on each stroke. Filing should be continued until the saw cuts are completely removed, and for a little while longer.

Emery Paper. The paper is placed on a sheet of glass, and the specimen is rubbed in a direction at right angles to the file scratches.

It is usual, at each stage in the polishing, to turn the specimen through 90° so that the polishing is always at right angles to the direction of the previous stage. Three grades of carborundum paper are employed in this way, beginning with a coarse paper, and finishing with a fine one.

Carborundum Powder. A horizontal wheel revolving at about 300 revolutions per minute is employed. It is covered with fine woollen cloth, which is wetted with water, and dusted with fine carborundum powder. The specimen is moved from centre to circumference and back again as the wheel revolves, and after a while is turned through 90°. This stage is complete when it is very difficult to see any striations with the naked eye.

Final Polishing. A second wheel is used, which is kept well wetted, and dusted with either floated emery powder, rouge, or calcined heavy magnesia; the magnesia gives the finest polish. It is very important at this stage to keep the specimen cool, and not to employ more pressure than is necessary.

Washing. Distilled water is used for washing, and after removal of the polishing material the surface is dried with lens paper. The surface must not now be touched by anything except lens paper until after etching.

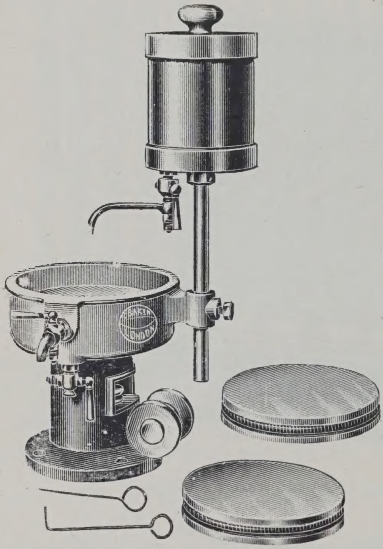


Fig. 47. Metal Polishing Apparatus (*Baker*)

Etching. The surface is dipped in the appropriate etching solution, of which a number is given in Chapter XVII. The etching is continued for a few seconds only, and repeated after examination of the specimen until the correct degree has been attained.

Surface flow, which falsifies the true structure, may easily take place even with iron and steel, but more especially with the softer metals, such as aluminium and lead. It may be prevented by keeping the specimen cool, during the polishing, and by moistening with paraffin in the earlier stages. The paraffin must, of course, be thoroughly removed before etching.

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CHAPTER IV

MEASUREMENT, COUNTING, AND DRAWING

QUANTITATIVE microscopy is largely concerned with measuring and counting, either directly on a preparation or on a drawing or photograph. Quantitative microchemistry is a subject outside the scope of this book, but those interested may read Pregl, *Quantitative Organic Microanalysis*; Donan, *Die Arbeitsmethoden der Mikrochemie*; and Emich, *Lehrbuch der Mikrochemie.*

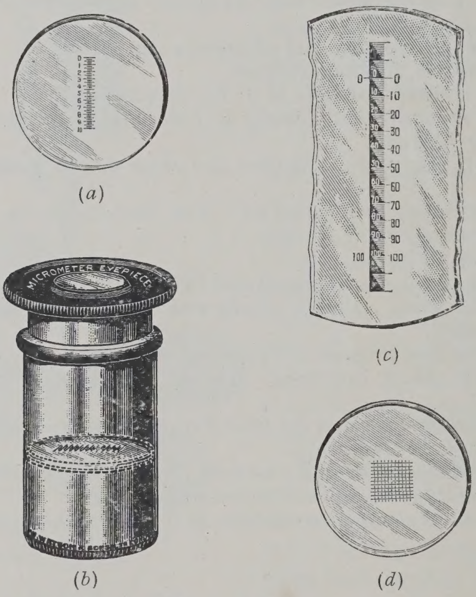

FIG. 48. EYEPIECE MICROMETERS

(*a*) = Micrometer scaled eyepiece.
(*b*) = Showing (*a*) in position in eyepiece.
(*c*) = Step micrometer scale (enlarged).
(*d*) = Net-ruled eyepiece scale.

Drawing. FREEHAND DRAWING. It is well to get into the habit of making rapid freehand sketches when only approximate accuracy is required. The easiest method of doing this is to employ a net ruled scale in the eyepiece, and to draw on squared paper (centimetre squares). The paper should be supported on a sloping board at about the level of the stage.

The magnification of the drawing should be determined beforehand by one of the methods given later. A low-power eyepiece, about ×5, will be found most convenient. A soft BB pencil is preferable to a hard pencil

CAMERA LUCIDA DRAWING. Freehand sketching is difficult for those with no aptitude for drawing, and at best it is not easy to obtain correct rendering of proportions unless painstaking and time-consuming measurements of the preparation are taken by means of an eyepiece scale. For this reason, a camera lucida is very convenient. There are two types; the one being employed with the microscope vertical, which is very convenient when preparations are being examined in a fluid medium, and which may be represented by the Swift-Ives pattern; the other being used with the microscope inclined at such an angle that the paper is normal to the camera lucida axis, of which type the Leitz model is illustrated.

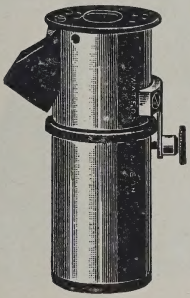

FIG. 49. CAMERA LUCIDA (*Watson*)

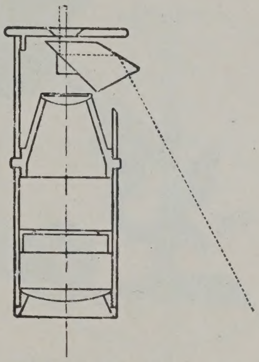

FIG. 50. PATH OF RAYS IN CAMERA LUCIDA

The chief difficulty encountered in using these attachments is the control of the relative illuminations of the field of view and the projected image of the paper and pencil which is seen superimposed on the field image. It occasionally happens that the relative brightness of the two images cannot be suitably adjusted, and the brighter image will then be found to swamp the other, making work very difficult; resort must then be made to freehand drawing. Graph paper is again very serviceable for the sketching, for if the instrument be set to a known magnification, the ruling of the paper provides a permanent and convenient record of the scale to which the drawing is made.

These eyepieces may also be used for such purposes as the projection of the scale of a thermometer into the eyepiece, when taking a melting-point on the stage.

Main outlines only should be put in with the camera lucida, detail being afterwards included by freehand drawing, when the image of the preparation is not confused by the projected image of pencil and paper.

Linear measurements. These should be made on sketches by adjusting the magnification of the drawing to a known value. Direct measurements may, however, be made of objects in the field of view by means of eyepiece scales.

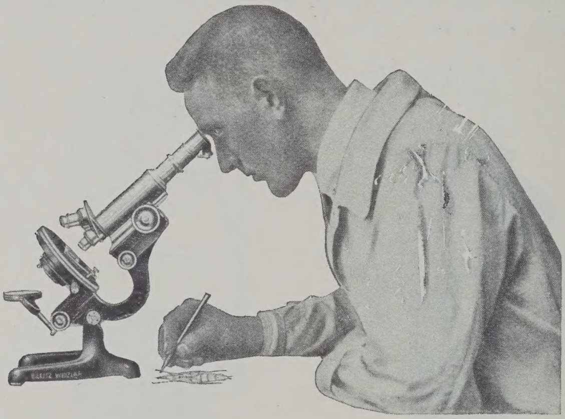

FIG. 51. CAMERA LUCIDA IN USE (*Leitz*)

The unit of measurement is the micron (μ), which is 1/1000 mm. or 1×10^{-6} metres. The millimicron, abbreviated as mμ (or sometimes, incorrectly, as $\mu\mu$) is 1×10^{-9} metres, and is thus ten times the length of the Ångstrom unit (A.U.), which is used for denoting the wavelength of light and is 1×10^{-10} metres.

The magnification given by a drawing from a camera lucida may be obtained very simply. A *stage micrometer*, which consists of a slide ruled in 0·1 and 0·01 mm., is placed on the stage and focused, e.g. by the 16 mm. objective. By means of the camera lucida, the markings as seen through the eyepiece are pencilled on a piece of plain paper; the distance apart of these lines is then measured by means of a ruler, in millimetres. The numerical magnification obtained is then obvious. It is preferable, however, to adjust the height of the paper, and the length of the draw tube, so that this magnification is not, for example, such a number as ×267, but a round number; convenient magnifications to adopt as standards are ×25, ×50, ×100, ×200, and ×400.

The magnification may be increased by increasing the length of the draw tube, for although this causes spherical aberration, the effect is not of great importance with the lower powers, for this particular purpose. The magnification can be decreased by raising the paper, thus bringing it closer to the eyepiece. The most suitable position for the drawing pad is in the same plane as the stage, but this is not a matter of consequence.

When graph paper is being employed, it is, of course, unnecessary to mark on the paper the stage micrometer units; the position of the paper, and the draw-tube length are adjusted until the markings coincide with the paper rulings.

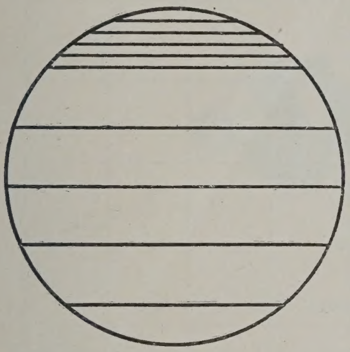

FIG. 52. STAGE MICROMETER SEEN THROUGH A 4 MM. OBJECTIVE

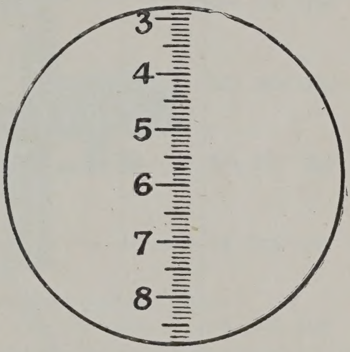

FIG. 53. EYEPIECE MICROMETER SEEN IN A × 10 EYEPIECE

Eyepiece scales. When drawing is not being brought into service it is often very convenient to take measurements of the preparation directly. This may be carried out by means of an eyepiece micrometer scale, which is dropped into the eyepiece (after unscrewing the top lens), where it rests on the diaphragm. It is an advantage to have a focusing ocular, which enables the markings to be brought into sharp focus, but the scale may be adjusted into the correct position in normal oculars, by building up on its lower surface thin rings of paper to lift it from the diaphragm to the necessary extent.

The evaluation of the eyepiece divisions is carried out by means of the stage micrometer. The markings on the latter are focused, and the whole length of the eyepiece scale is read off in one-hundredths of a millimeter. Simple division then gives the value of the ocular scale divisions. It has often been recommended to adjust the tube length so that the magnification is a round number, but in the writer's opinion the advantage of using the correct tube length (whenever drawing by camera lucida is not being attempted) far outweighs any slight convenience gained by the altered tube length. It is a simple matter to draw up a graph in which eyepiece divisions

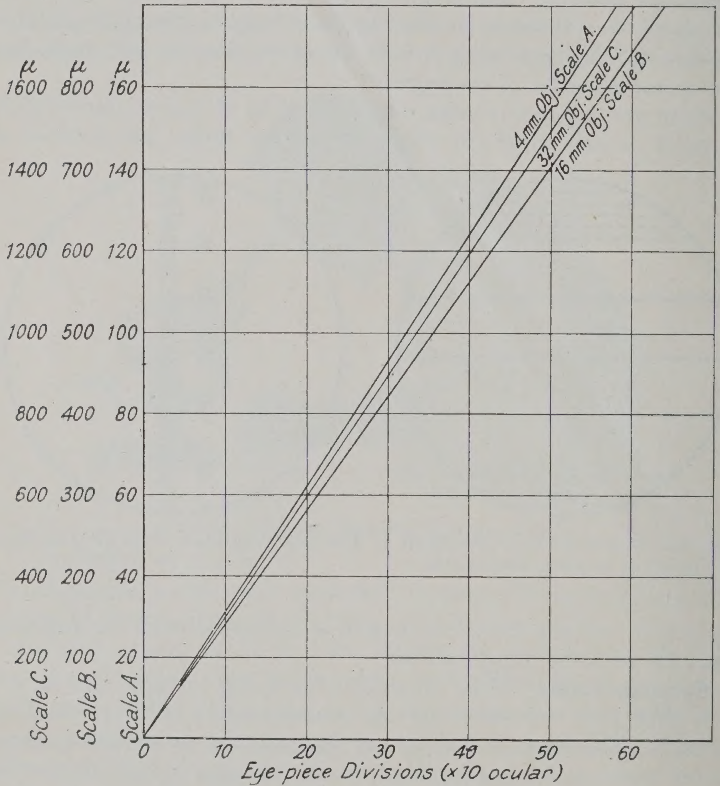

FIG. 54. GRAPH GIVING MEASUREMENTS IN MICRONS FROM EYEPIECE SCALE READINGS

are plotted against the length represented, and to consult it when necessary. A very useful eyepiece scale for exact measurements consists of a cross-hair which can be moved across the field, the distance moved being recorded on an external vernier.

EYEPIECE NET MICROMETER. This most useful accessory is an eyepiece scale, 1 cm. square, ruled into 100 squares. It is a great convenience for freehand drawing on squared paper, and is especially useful with low powers. When areas are to be measured on a photograph, it may be projected with the image. The calibration of the divisions is carried out in the usual way, by the aid of the stage micrometer. (See Fig. 48.)

CONDENSER MICROMETER. It is possible by means of the condenser to project an image of a scale into the plane of the preparation, the scale and the image being seen simultaneously. It has the advantage that the scale does not move in relation to the object, when the eye is moved slightly (which is a point to be guarded against with the eyepiece scale). On the other hand, the stage often has to be rotated in order to bring the specimen into a suitable position for measurement with the scale, which is sometimes inconvenient if the stage is not centred exactly; the eyepiece micrometer can, of course, be rotated without the specimen being affected.

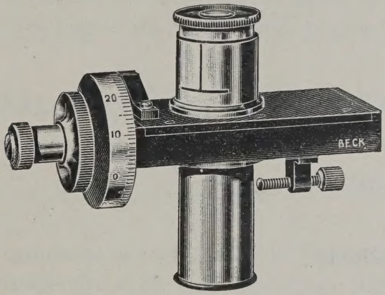

FIG. 55. COBWEB EYEPIECE MICROMETER (*Beck*)

Photographic magnification. The magnification of photographs is determined by projection of the stage micrometer on to the ground glass of the camera, where it is measured by means of a ruler. It is not always convenient to adjust the magnification to a round number in this case, and a useful method of enabling the magnification to be estimated even for example, after a photograph has been enlarged, is to make on a cover glass (which is placed in the eyepiece) two marks by means of a diamond, at the extreme edges of the field; these are recorded together with the image on the negative, and as their value is easily determined by the above method, the exact magnification of any print may be calculated.

Area measurements. The measurement of areas often provides data from which volume and weight proportions of constituents of the preparation may be estimated.

1. FROM A DRAWING ON SQUARED PAPER. The area of any section of the preparation is computed by counting the squares covered,

considering squares less than half occupied as nil, and squares more than half covered as one.

2. By Planimeter. This well-known instrument is very convenient when it is required to estimate the total area of a number of irregularly-shaped structures on the drawing or photograph.

Volume proportions are easily obtained when the thickness of the section examined is constant. Weight proportions are obtained in this case when the specific gravities of the constituents are known. For example, if a cross-section of a yarn is being observed, which is composed of a mixture of silk (S.G. 1·3) and artificial silk (S.G. 1·5), and the total areas in the preparation are relatively, silk 20, artificial silk 40, then the weight percentages are found as follows—

$$\text{Silk: } \frac{20 \times 1{\cdot}3 \times 100}{20 \times 1{\cdot}3 + 40 \times 1{\cdot}5} = 30{\cdot}3\%$$

$$\text{Artificial silk: } \frac{40 \times 1{\cdot}5 \times 100}{40 \times 1{\cdot}5 + 20 \times 1{\cdot}3} = 69{\cdot}7\%$$

Counting Methods. The Wallis lycopodium method is very useful, and makes use of the fact that the spores of *Lycopodium clavatum L.* are very constant in size and weight, 94,000 spores weighing 1 mg. It can be carried out with almost any objective, and no special apparatus is required. The method consists, in brief, in mixing a definite weight of spores with a definite weight of the substance under examination, suspending the mixture in a suitable medium, and making a count of the number of particles of the substance per lycopodium spore in the field of view.

About 0·1 grm. of spores are mixed carefully with about 0·1 grm. of the substance, in a small, shallow watch-glass. A little of the suspending agent (which may be either a mixture of castor and olive oils, or gum tragacanth solution) is added, well mixed in, and the remainder of the suspending medium added in small portions with thorough mixing, until a sufficient volume of medium has been added to give only 10 to 20 spores in the field of view. This volume, using 0·1 grm. of spores, and a 4 mm. objective, is about 20 cc.

Two or three mounts are now prepared by taking just sufficient of the suspension as will fill the space under a cover glass, and mounting it for observation. The particles of substance in the field of view, and also the spores present, are counted for ten different fields on each slide; these fields should be chosen to represent most parts of the mount, but should not be too close to the edge. Two slides at least should be counted in this way. The counts of the ten fields should be added together for each slide, and the number of particles (n) per 100 spores ascertained for each slide by the following calculation—

$$n = \frac{\text{Number of particles of substance} \times 100}{\text{Number of lycopodium spores}}$$

The figure n obtained for each slide should be about the same. These are then averaged, and the number of particles of substance per milligram N is obtained by the following calculation—

$$N = \frac{n \times 94{,}000 \times L}{100 \times S}$$

where S = weight in grammes of the substance taken.

and L = weight in grammes of the spores taken.

This method finds its chief application in the analysis of mixtures of substances, such as two or three kinds of starch, which cannot be analysed, or are not easily analysed, by ordinary methods.

The method is carried out by first making a "standard" mixture of the two substances A and B, in exactly equal proportions by weight. The standard mixture thus obtained is counted as above described, and a similar count is made on the sample of unknown composition, in each case of the particles of A. By calculation, the number N for both specimens may be obtained, and

$$\% A \text{ in sample} = \frac{50 \times N_{\text{sample}}}{N_{\text{standard}}}$$

or (avoiding three calculations)

$$\% A \text{ in sample} = \frac{50 \times n_{\text{sample}} \times L_{\text{sample}} \times S_{\text{standard}}}{S_{\text{sample}} \times n_{\text{standard}} \times L_{\text{standard}}}$$

If the same weight (e.g. 0·1000 grm.) of sample and of standard are each mixed with this weight of spores, the calculation simplifies itself into the expression—

$$\% \text{ of } A = \frac{50 \times n_{\text{sample}}}{n_{\text{standard}}}$$

The method is of wide application, especially since the spores are very resistant to reagents, and may be mixed with a specimen of flour for example, from which crude fibre is to be prepared.

Particle size. The influence of the size, aggregation, shape, and uniformity of particles of substances employed in industrial operations, on the course of the process, has within recent years become of more and more interest. All the methods available for determination of these properties are rather tedious: the first to be described (due to Zsigmondy) is a modification of the one already discussed in connection with the slit ultramicroscope, and is suitable for particles of constant size: the second method (by Perrott and Kinney) is capable of dealing with specimens containing particles which differ largely in size.

1. A flat cell, such as the haemacytometer cell used for blood counts, is filled with a suspension of a known weight of substance in a known volume of one of the suspending media described. Dark ground illumination is employed; the counting is facilitated if the particles are allowed to settle in the cell for some time before commencing the observations. The calculation has already been given on page 50.

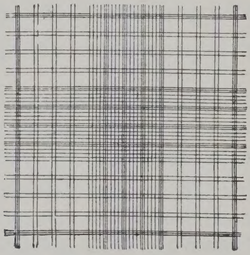

FIG. 56. HAEMACYTOMETER CELL (*Baird and Tatlock*)

2. The same cell is employed as in the previous method, but several of the squared areas are counted in the following way: The size of each particle counted is computed, and after some 200 particles have been recorded (covering at least ten fields), a size-frequency curve is plotted. This at a glance gives a picture of the uniformity of the substance, from which the much more useful average particle weight (or particle volume, if of more interest) curve may be deduced.

Comparison with standards. When mixtures of two or three components are continually being examined for percentages of the components, the preceding counting methods may be replaced by the much quicker, though less accurate, method of comparison with a series of perhaps half a dozen previously prepared standard mounts which cover the possible proportions. The accuracy attainable with practice is perfectly adequate for a great many industrial requirements.

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CHAPTER V

PHOTOMICROGRAPHY

THE microscopist will always, as far as possible, rely upon actual mounted specimens for reference purposes. In many cases, however, permanent mounts are for various reasons impossible, and recourse must then be made to some form of drawing or photograph. For reproduction and illustration purposes, and particularly for the provision of a rapid and convenient means of refreshing the memory upon various points, records are no less valuable.

Drawing possesses certain advantages over photography. It rarely happens that all the characteristic features of a preparation are found in the same field of view, whilst the special details of a large number of preparations may be combined in one sketch. On the other hand, drawing requires a natural aptitude in the first place, and is even then tedious and trying to both eyes and temper.

Photomicrography, systematically carried out, in addition to being very rapid, is within the reach of anyone who will pay careful attention to detail in manipulation, and this is implied in all successful microscopic work. The results, properly interpreted, are very accurate, and far more vivid than any drawing can be.

Photomicrographic apparatus may be either horizontal or vertical, and each form has its advantages. Every worker who has much photomicrography to carry out, sooner or later adopts the vertical form as being more convenient and far less tiring than the horizontal type. The horizontal form is somewhat more free from vibration, which is an advantage with high magnifications. Further, a long bellows extension is easily obtained, allowing very large magnifications to be recorded with low-power objectives, when, as occasionally happens, such magnifications are required.

The vibration bogey is, however, greatly exaggerated. Vibration due to trams or traffic on a road outside the laboratory, for example, will have a negligible effect; on the other hand, a book dropped on to the table may cause trouble by moving the slide closer to the stage. For the same reason, it is always wise to focus upwards for photographic work, as there is then no chance of the body tube dropping slightly during the exposure.

There are numerous manufactured forms of apparatus on the market, horizontal and vertical. The vertical apparatus usually consists of a tripod carrying the camera over the microscope, but

Leitz have a model which fits on to the microscope draw tube, and is excellent in every way for general work, even at high magnifications, provided that the microscope is sufficiently rigid in its adjustments.

The horizontal form may be made very simply by taking out the lens from an ordinary bellows camera, and connecting up the microscope in any convenient manner. Fig. 58 shows an apparatus used for some years by the author.

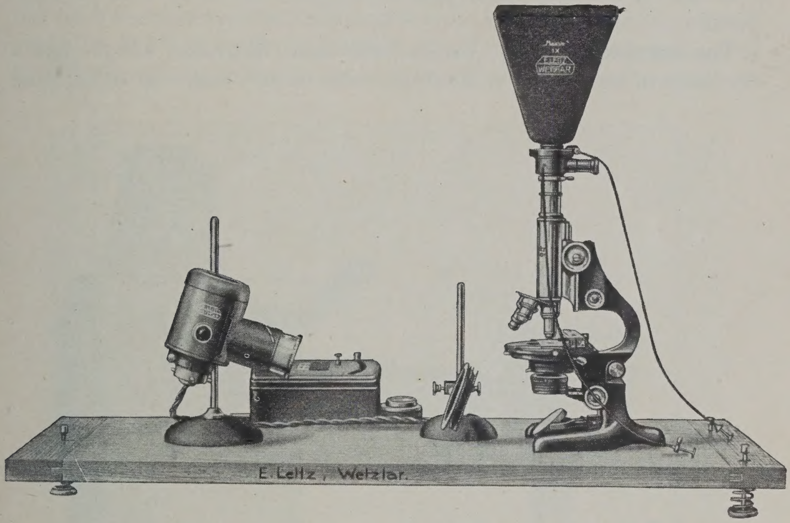


Fig. 57. Photomicrographic Attachment (*Leitz*)

A brass tube with a screw thread on one end screws into the camera lens opening in the camera. It is about 1½ in. long, and the inside diameter is large enough to take the eyepiece end of the microscope tube easily. The connection is made light-tight by means of a tube of velveteen, double thickness, which has a rubber ring sewn in at each end. The microscope is clamped by its base on to a thick piece of wood, and the camera raised by a further block of wood screwed on at such a height that when the arm of the microscope is swung into the horizontal position the brass tube is at the correct height for connection as described. The board is supported on four thick rubbers which act as shock absorbers, and which may be screwed on through holes which are deeply countersunk in the rubber. Fig. 59 shows the method of clamping down the microscope base.

The distance at which the plate is placed from the ocular depends on the magnification required, but 9 or 10 in. will be found suitable as a general rule. The diameter of the field at this distance is conveniently such that a quarter-plate occupies the centre portion only, the edges of the field, where any distortion may be present, being thus outside the photograph.

A few of the important points may now be dealt with in more detail.

The microscope tube. Various textbook diagrams which depict the path of light rays in the body tube would lead one to suppose that no light falls on the sides of the tube. Actually, however, an appreciable amount of light leaves the objective at such an angle that it does fall in this way, and is reflected thence into the ocular, to an extent which depends upon the reflective power of the walls, and the diameter of the tube. This reflected light causes the image to be somewhat fogged, because it dilutes the dark parts with white light, and thus lessens the contrast. The effect is undoubtedly most marked in photographic work, but if suitable precautions be taken to prevent this stray light from entering the eyepiece, a decided improvement in the clarity of the visual image also may be obtained.

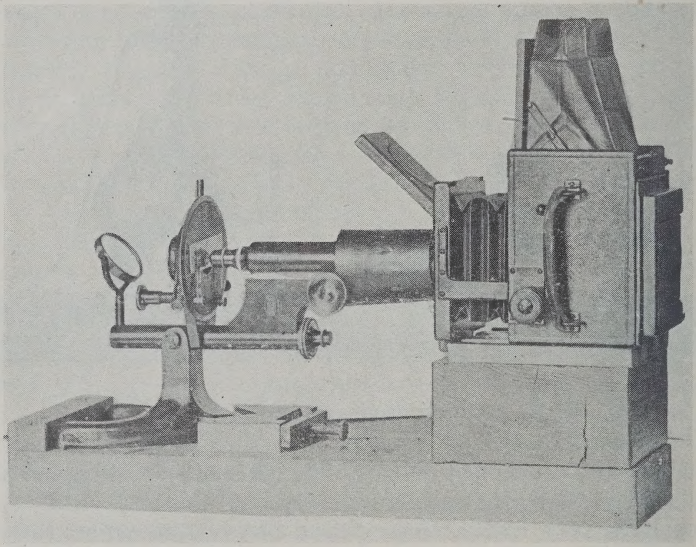


FIG. 58. SIMPLE PHOTOMICROGRAPHIC APPARATUS

The usual method adopted by the makers of microscopes intended for much photomicrography is to construct the body tube with a much larger diameter than usual. The advantage gained in this way is considered by many experienced workers to be somewhat doubtful. An excellent result may, however, be obtained in a very simple way by placing a diaphragm in the tube, the opening of which is as small as it is possible to employ without interfering with the field diameter. This diaphragm is constructed from a piece of fairly thick black cardboard, which is cut into a disc of such a size that it fits tightly inside the draw tube. The cardboard having been cut, its centre is found, and a circle of 15 mm. diameter is cut exactly in the centre. The diaphragm thus made is now pressed down about 2½ in. into the draw tube, from the ocular end; two or three experiments will give a distance down the tube which will show a noticeable improvement in the contrast of the photographs.

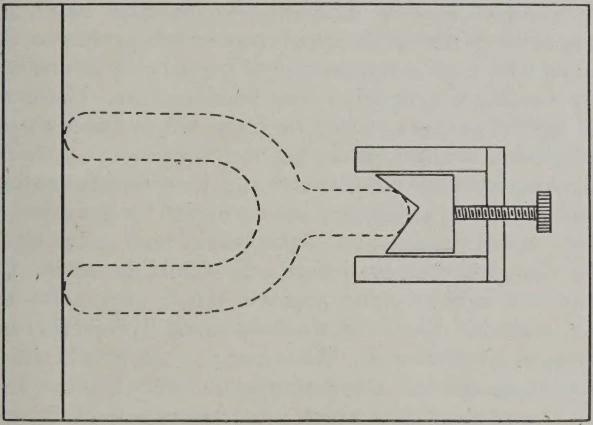


FIG. 59. DIAGRAM OF CLAMPING ARRANGEMENT FOR MICROSCOPE IN FIG. 58

Unfortunately, all types of microscopes do not lend themselves to the application of this device, owing to small peculiarities of construction. A small stop of a similar character cut out of black paper may, in this case, be placed at the lower end of the draw tube or inside the tube of the objective. With some objectives, which have a very convex back lens surface, it may be found that this stop is of advantage when the draw-tube diaphragm is also employed. The actual dimensions of these stops will, of course, vary with the instrument. (See Fig. 16.)

With the same object of preventing any loss of contrast by reflected light, the bellows of the camera should be large compared with the size of the plate to be used; thus, a camera capable of taking a half-plate should be used only with a quarter-plate. Some forms of apparatus sold are ridiculously inadequate in this respect. Small bellows, whilst they rarely actually block out any of the field, undoubtedly give rise to internal reflections which cause this fog or haze of the image. It may even be advisable, when the bellows are rather narrow, to have stops in the interior of the camera.

Objectives. Depth of focus is a very desirable quality in an objective for photomicrography, as an accurate photograph of a fairly thick object may be obtained. On the other hand, as depth of focus is incompatible with a high numerical aperture, and, therefore, usually with high magnification, it must be sacrificed when the purpose is to obtain a record of very fine structure. Unfortunately, even the best objectives cannot be corrected in order to obtain a flat field of view without lessening the definition at the centre; individual requirements will, therefore, react on the choice of an objective. Aplanatic objectives are corrected for spherical aberration, which causes the outsides of the field of view to be out of focus when the centre is sharply defined. It should be added that the flatness of the field of view depends largely upon the eyepiece employed, and that there are specially made "projection oculars" for photomicrographic work. With low powers, it is often possible to take photographs of sufficient magnification without an ocular (many of the photographs which illustrate this book have in fact been so taken), but it should be remembered that only the centre portion of the field is not noticeably distorted because, in effect, the tube length of the microscope is considerably increased beyond that for which the objective was designed. The two chief results are a different focus from that obtaining with an eyepiece, and a certain amount of spherical aberration.

A much more important point is the chromatic aberration, for which achromatic objectives are specially corrected. Chromatic aberration causes the image to have coloured fringes, and objectives are usually corrected to bring two spectrum colours to focus in the same plane. This type, excellent for visual work, introduces serious difficulties in photomicrographic operations, since the chemical and visual foci lie in different planes. In consequence, a photograph of an object which is in perfect focus visually will appear blurred. To overcome this difficulty, and also to produce images which will show no trace of chromatic aberration, apochromatic objectives have been introduced, which are, however, usually four or five times as expensive as the achromatics. They bring three spectrum colours to focus in the same plane, but as the images are not congruent, special "compensation oculars" must be employed. The field obtained is usually not flat. (See also Chapters I and II.)

Control of contrast. It now remains to discuss the methods by which detail visually observable in a mount, may be reproduced on a photographic plate. This is by no means so simple a matter as might at first appear, and, in fact, the control of contrast is the greatest problem in microphotography.

Contrast may be of two kinds, which require different treatment: the differentiation of internal structures, such as a cross-section of a vegetable tissue (stained or unstained); or the sharp rendering of an almost transparent object against its background, diatoms being a good example of this type of subject.

OUTLINE CONTRAST. The second type of specimen is successfully photographed only by paying careful attention to critical illumination, which is the most important single factor. Contrast may be accentuated by giving as long an exposure as the plate will take safely, by using a "process" plate, and by printing on a very contrasty paper. If resolution can be sacrificed, the apparent contrast may be increased by using a mounting medium, the refractive index of which differs fairly considerably from that of the specimen. A similar effect is obtained to a lesser degree by the use of white light, or, in other words, a colour filter should not be employed.

INTERNAL CONTRAST. The first group of objects mentioned is the larger and more important. In its simplest form the specimen is of one colour only, e.g. a red-dyed textile fibre. White light may in this case be employed, with only a slight loss, not of contrast, but of resolution.

If, however, only part of the object is coloured, for example, a red-stained botanical section, white light, although to some extent satisfactory, would not give the best results. Two alternatives are possible, the employment of a colour screen of the *same* colour as the stain (red), or of the *complementary* colour, in this case, blue-green.

As already explained, a blue-green filter would cause the red-stained portion of the section to appear black on a much less pronounced ground. The general shape of the stained tissue would stand out very plainly, but no detail would be observable in it. The light "transmitted" through the object thus tends to be entirely absorbed, the object being photographed as though it were black on a white background.

If a red filter of exactly the same absorption as the stain be employed, theoretically, and to a very considerable extent practically, there would be almost no differentiation between the stained and unstained portions of the section on the resulting photograph. On the other hand, if the absorption bands of the red screen do not exactly coincide with those of the stain, being either to one side, or broader, the red portion would stand out as a darker grey against the remainder of the section, by reason of the absorbed light; internal detail would, however, be observable, because the light would not be absorbed so completely that the photograph would show the red as black.

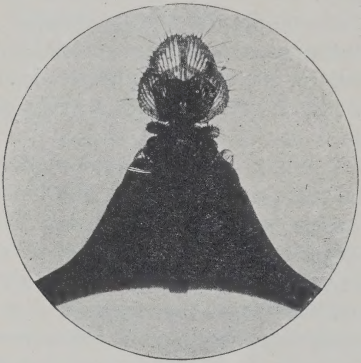


Fig. 60. Photograph Taken to Obtain Contrast

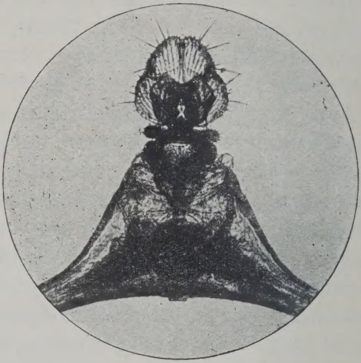


Fig. 61. Photograph Taken to Obtain Detail

The rules governing the choice of colour screens may, therefore, be summarized as follows—

For contrast: Use a screen of the complementary colour to the stain, transmitting light of a wave-length within the absorption band of the colour of the specimen.

For detail: Use a screen of about the same colour as the stain, but not quite; it will then transmit light mostly of a wave-length lying outside the absorption band of the stain, but also will allow some light to pass within this absorption band.

In many instances, preparations are stained in two colours, usually complementary to each other. The colour of light used for such objects is decided by the relative importance of the stained tissues, and the amount of detail required. A general reproduction of the visual appearance may often be obtained in monotone by means of white light and a panchromatic plate.

In the case of a specimen stained red, counterstained green, the use of a red filter would cause the green to appear black on a light grey ground, whilst a green filter would show the green-stained tissue as a light grey upon a black ground. Neither of these appearances is very useful; the ideal method is obviously to employ a colour screen with an absorption curve showing a wide band in the

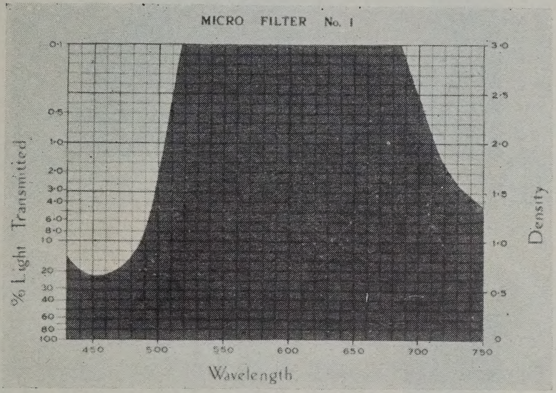


FIG. 62

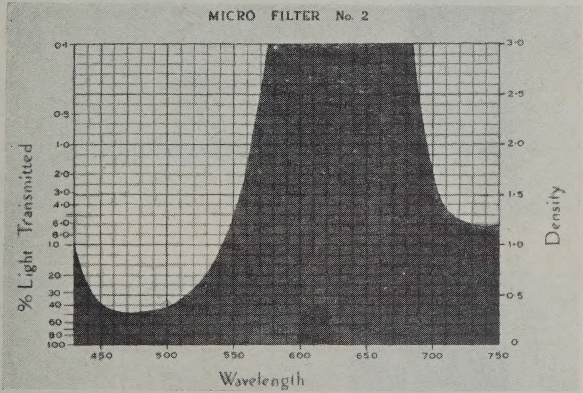


FIG. 63

green, and a smaller band in the red region of the spectrum; the resulting photograph would indicate the red-stained tissue as a dark grey on a light grey background. The choice of the correct screen is in such cases a rather difficult and complicated matter. In the first place, the dyes employed as micro-stains themselves often deviate from the ideal absorption curve, which shows a single narrow band. Often two bands of unequal strength are present,

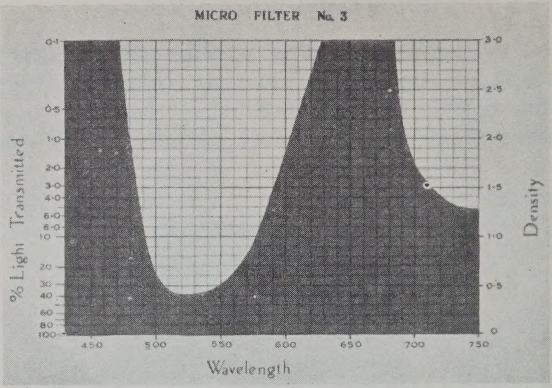


FIG. 64

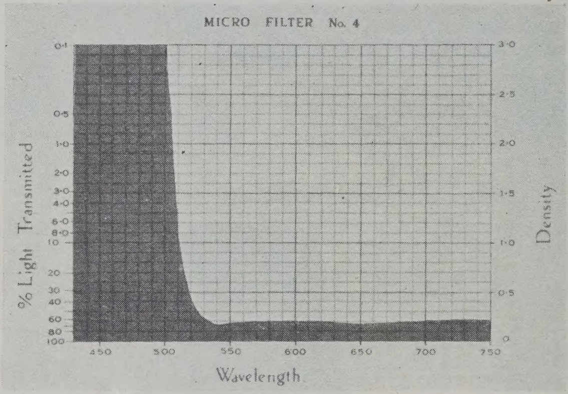


FIG. 65

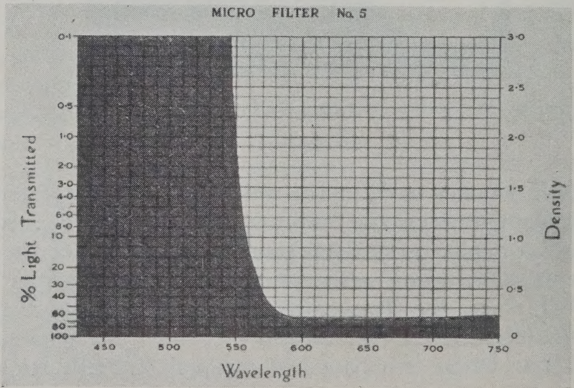


FIG. 66

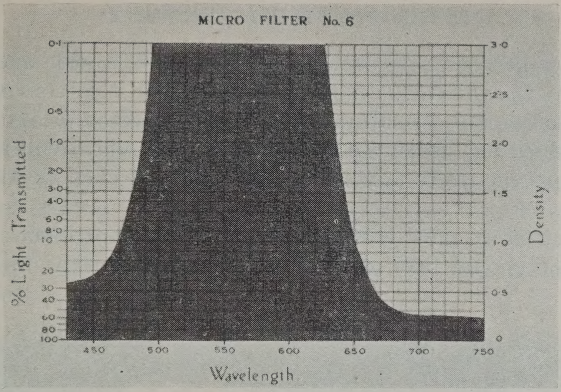


FIG. 67

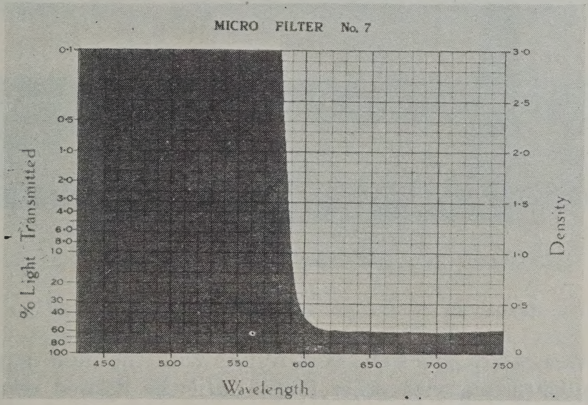


FIG. 68

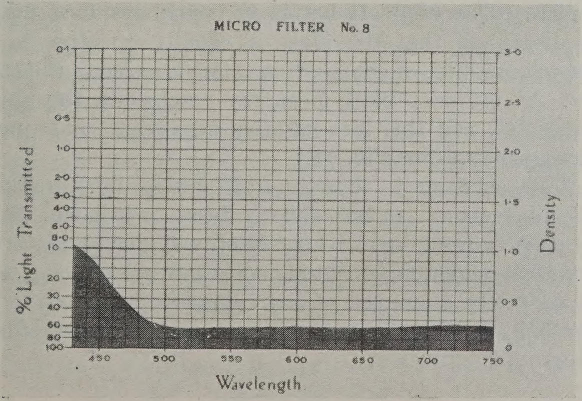


FIG. 69

whilst in some instances, such as haematoxylin, a wide and diffuse absorption is exhibited. In the second place, it is convenient in practice to employ a small number only of selected colour screens, each of which has a definite transmission, but which will rarely be exactly appropriate to any stained preparation, even when used in pairs.

The best screen must therefore be chosen by experiment, and the preparation should first of all be examined under the microscope with a filter which gives the best results with the more important tissue, pairing this with other filters until the desired relation of contrast is obtained. (See also page 15.)

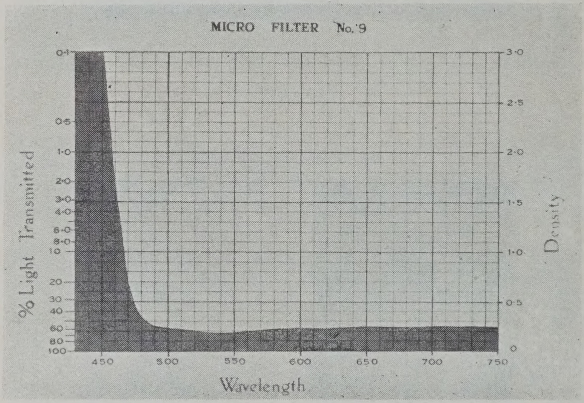


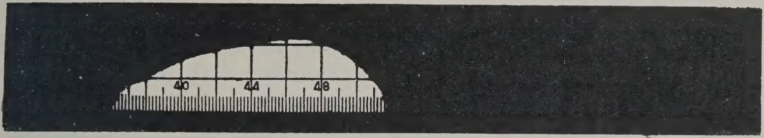
FIG. 70

Plates. So far, no mention has been made of the type of photographic plate to be used. It has been tacitly assumed during the previous discussion that the plate is equally sensitive to all the spectrum colours, but, of course, this is far from being the case with all plates. An ordinary plate is mainly reactive in the blue-violet and ultra-violet, and this fact has two important consequences in photomicrography. (See Fig. 71.)

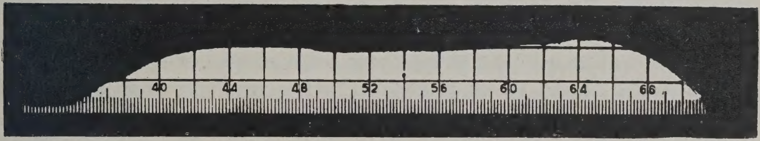
First of all, achromatic objectives are not in photographic focus when visually correct, as has already been mentioned. Secondly, a stained preparation taken without filter, will give totally erroneous colour renderings in monotone, red and yellow reproducing as black; whilst if a filter be employed of any colour from the red to yellow-green, the light passing through will have practically no effect upon the photographic emulsion.

In order to overcome the first difficulty, four methods are available.

(*a*) A colour screen may be used which cuts out the ultra-violet whilst not interfering with the visual spectrum, and more particularly with the blue-violet. The exposure is, however, lengthened, and, furthermore, because the eye chiefly uses the orange-yellow-green of the spectrum for focusing, there is a tendency for the photograph to be still out of focus unless the objective is suitably corrected.



(*a*) Ordinary plate



(*b*) Panchromatic plate

FIG. 71. SENSITIVITY OF PHOTOGRAPHIC PLATES (*Ilford*)

(*b*) The fine adjustment may be turned to an extent ascertained by trial, until the negative becomes sharp. This value then becomes a constant for that combination of objective and ocular.

(*c*) The ordinary plate may be replaced by a panchromatic plate, which is sensitive to all colours, and a filter, such as the orange Ilford delta employed with it, in order to obtain light from a narrow spectrum band. The only disadvantage in this case is that panchromatic plates are more troublesome than ordinary ones because they must be developed in total darkness, but the method may be recommended strongly.

(*d*) The most satisfactory solution to the problem is to replace the achromatic objective by an apochromat, which brings the ultra-violet image practically into the same plane as the yellow image.

The second disadvantage of the ordinary plate, that it will not render colour values properly, can only be overcome by the use of a panchromatic plate. A panchromatic plate, however, is also sensitive to the ultra-violet, and peculiar results may occasionally be obtained when no filter is employed with a stained preparation, if one of the stains has an "invisible" transmission in the near ultra-violet region. A filter cutting out the ultra-violet should, therefore, be employed. Apart from this, however, the plate may be largely left out of one's calculations whatever filters are employed, and whatever the colour of the preparation. The exposure to be given is, of course, always lengthened by the employment of filters.

Photographic manipulations. The remainder of the questions to be dealt with are purely photographic, and are: focusing, exposure, development, and printing.

Focusing. The image may be focused on ground glass in the ordinary way, but better results are obtained by other methods, especially when high magnifications are being employed, and the light intensity is low. The transparency of the ground glass (which should be as fine-grained as can be obtained) may be increased by dissolving a little vaseline in petroleum ether, and allowing the glass to dry after immersion. If the vaseline is rubbed on, it is difficult to avoid small specks of cloth, etc., adhering to the glass, and, furthermore, the vaseline can never be applied without remaining streaky. The streakiness can be made less pronounced by carefully holding the glass above a small flame for a second or so.

When the object to be photographed is small in area, a drop of Canada balsam, placed in the centre of the ground glass, may be "mounted" by a thin cover glass, thus producing a transparent patch. The image cannot be focused on this patch unless some form of adjustable focusing magnifier is employed. A suitable one is supplied by Watsons, and may be focused on a speck of dirt on the underside of the ground glass, being then firmly fixed at this focus. When large areas are to be photographed, an old negative, which has been well cleaned, may be substituted for the ground glass, focusing being carried out by means of a focusing glass, as before. In cold weather, the glass may become misty owing to moisture from the breath condensing, but a little dilute glycerine or weak soap solution smeared over the ground glass and wiped off will prevent this.

The image on a ground glass screen may be magnified by any magnifier, focusing or not, because the plane of the ground glass is observed, whereas with a plain glass, the plane corresponding to the exact focus of the magnifier is seen. Using a ground glass, therefore, a single lens from an eyepiece, or even some older types of 1 in. or 2 in. objectives, may be employed as focusing glasses.

Exposure. The chief factors affecting the length of exposure are the speed of the plates, the colour filter employed, the intensity of the light source, the size of the condenser stop, the objective and ocular used, and the thickness of the slide.

The plate may be chosen according to the contrast of the subject.

Extremely contrasty preparations require a plate with a large tone range, such as the Ilford Process Panchromatic, whilst a specimen with very little contrast may be taken on a portrait plate. The average microscopic object takes very well on the Ilford Special Rapid Panchromatic plate. However, the great majority of plates have a wide tone range, and, provided that the correct paper is used for printing, the only property which need be considered is its speed. When illumination is very poor, very fast plates may be employed, but as a rule, a fast plate shows more "grain" than a slow one, which is a disadvantage.

The colour filter multiplies the exposure necessary without filter, by a factor which varies from 2 to 10, but which is usually stated by the maker of the plates or filters. This factor applies only to panchromatic plates; a red filter may increase the exposure of an ordinary plate as much as 900 times.

The ideal light source is a point of light of great intensity, but for most purposes, and especially for low-power work, an ordinary opal globe may be employed successfully. It should be focused by means of the substage condenser in the plane of the image, as usual, and thrown slightly out of focus in order to prevent the grain of the opal glass from being seen.

The condenser stop should be adjusted as for visual work; if too small, resolution is impaired, if too large, glare is introduced.

The thickness of the slide has considerably more effect on the exposure than might be realized, and for permanent mounts only those slides should be used which are of the thickness recommended by the maker of the substage condenser.

It is obviously very necessary to reduce these factors as far as possible to a standard, if exposures are not to be merely guesswork. This may be done by adopting a constant light source, by always using one make and type of plate, by paying special attention to the condenser stop, and by using the correct thickness of slide. In this way, the only important remaining variables are the colour filter, and the magnification. Here, again, the colour filter effect is known and can be allowed for by factors, whilst it is a simple matter to use one eyepiece, say × 8, and one length of bellows extension, leaving the objective as the only true variable. It then becomes a matter of experiment with each objective to find a suitable average exposure.

This should be such that a preparation with very little contrast, and a preparation showing considerable contrast should each, with this same length of exposure, give good negatives, in which the high lights are not clear gelatine, and the shadows are not dead black but show detail. One of these negatives, in the ordinary photographic parlance, will be "thin," the other "dense," but each will give equally good prints on the correct paper.

By adopting this method, the whole question is reduced to, say, four times of exposure, one each for the 32 mm., 16 mm., 4 mm., and the 2 mm. This system cannot be too much recommended; it is rather troublesome, in the first place, to find the correct exposures, but well worth while.

Development. All negatives, whatever the exposure, are given the same length of time in the developer, so-called under- and over-exposed negatives being given the same treatment. The time of development is decided only by the developer used, and the temperature of the solution.

The developer employed is largely a matter of choice, and there are many excellent formulae given in the photographic manuals. It is wise, however, to choose one formula, and use it to the exclusion of all others, becoming thoroughly familiar with its idiosyncrasies. An excellent developer, recommended by Dr. Glover, and used for some years by the writer, is the standard Kodak metol hydroquinone formula (No. 93). The time of development at 18° C. is 3 min. This developer is suitable for all plates and flat films, but because it is sensitive to dissolved oxygen it is not to be recommended for tank development of film packs or roll films. Film packs are very convenient, by the way, and can be obtained with a panchromatic emulsion.

For tank development, the glycin formula No. 96 can be recommended. The time of development at 18° C. is 20 min.

Panchromatic plates require to be developed in complete darkness. They may be desensitized by means of a solution of Pinacryptol Green or some such substance, but it is hardly worth the trouble, because one quickly becomes accustomed to working in absolute darkness, and times may be read on a watch with a luminous dial. A special green safe light may be employed, but there is always a danger of the plates becoming fogged.

After development, the plates are washed for a few seconds in clean water, immersed in the fixing solution (No. 97) until the creamy appearance has completely gone, and for 10 min. afterwards. They are then washed well in running water for at least half an hour, and allowed to dry at the room temperature, in a place where there is no dust flying about.

Printing. According to the character of the negative obtained, the printing paper is chosen from the various grades of gaslight or bromide paper.

Very thin negatives require very contrasty gaslight paper.

Thin negatives require soft or medium contrast gaslight paper.

Average negatives require vigorous bromide paper.

Dense negatives require a soft bromide paper.

One of these four papers will give a good print of the correct degree of contrast from any negative which is not hopelessly under- or over-exposed, that is, from any negative in which there is no clear glass or black shadow.

Thus, any one preparation may be taken with an exposure which produces a thin negative, and again with such an exposure that a dense negative is obtained. Each of these negatives, if printed on the correct grade of paper, will give prints almost indistinguishable from each other.

The printing may be carried out by any convenient light, such as an electric globe or an incandescent gas light. The printing frame should always be set at the same distance from the light, in order that the exposure may be gauged and controlled with ease. This distance may be 4 ft. for bromide papers, and 18 in. for gaslight paper. The exposures should then for most negatives lie between 5 sec. and 1½ min.

The actual exposure should be decided by trial. The printing frame, covered by a piece of cardboard, is placed at the correct distance from the light, the cardboard is removed, and after, say, 5 sec., one-fifth of the frame is covered up by the cardboard. After 10 sec., a further fifth is covered, and so on for exposures which increase in geometrical proportion, in this case 20, 40, and 80 sec. After a little practice it is only necessary to give three exposures.

Development of Gaslight Paper. The trial print is developed for exactly 30 sec., the temperature of the developer being between 55° and 65° F. One of the bands on the print will be considered the best, and the proper print should be made with this exposure, being developed exactly as before. (No. 95.)

The system of employing a fixed time of development, and selecting the exposure which best suits this time, automatically makes allowances for differences in the temperature of the developer and the speed of the paper.

Development of Bromide Papers. The trial print is placed in the developer, and the time, in seconds, is noted up to the first appearance of the image. This time is multiplied by five (using the M.Q. developer 94); and development is carried on for this total length of time. There is a possibility of error when a wide range of exposures is employed, as, for example, when the correct exposure is 5 sec., and an 80 sec. has been included, but a little practice will enable this to be discounted.

The "factorial" system of development automatically makes allowances for most of the variable factors in the exposure and development of bromide papers.

A few general notes on development may be useful. The print should, before being placed in the developer, be immersed for ½ min. in water, in order to wet it well. This water should have been stored in a jug in the dark room for some hours to ensure that it is at the same temperature as the developing solution. Fresh developer should be used for each print, and thrown away after use. From 15 to 20 cc. of developer are ample for a quarter-plate print.

When a print is—

Thin: increase the exposure.

Dark: decrease the exposure.

Flat, no contrast: the wrong paper has been used; change to a more contrasty paper.

Hard, too contrasty: again the wrong paper has been used; change to a softer paper.

FIXING OF GASLIGHT AND BROMIDE PAPERS. After development, the print should be placed instantly into clean water for a few seconds, and then into the fixing solution, "acid hypo." (No. 98.)

A large dish is essential, in which a number of prints can be placed clear of each other. The prints should be kept moving in this bath for 20 min., and afterwards washed in running water for about an hour. A good method is to pin the prints by one corner to a board, which is inclined at a slight angle, down which water is running, the board being made into a channel by pieces of wood along the edges.

Drying is carried out, after blotting the prints between the folds of a towel, in a moderately warm room.

The photographic method above outlined is the one employed by the writer. Any reader not familiar with photographic routine should consult two booklets by Dr. Glover, *Print Perfection* and *Perfect Negatives.*

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CHAPTER VI

MICROCHEMICAL TECHNIQUE

Dealing with small quantities of material. It should not be supposed that the technique of microanalysis is applicable only to those cases which fairly often occur in any chemical work, when a quantity of material is obtained which is too small to be treated, with any hope of success, by the usual laboratory methods. Owing to the small quantities of substance treated, the various operations are carried out far more rapidly than on the comparatively large scale of the ordinary laboratory process.

Acquaintance with the technique to be described will save a great amount of time and labour even in cases where the microscope itself need not be, and is not, employed.

The methods of microchemical work are only in a few cases different in type from those familiar to every chemist, being mostly modifications or adaptations of well-known processes or apparatus. In the majority of them, the microscope itself is not employed, for the object is to obtain the substance which is under examination either isolated from other matter, or in a suitable form for further examination by tests which are carried out with the microscope.

In the paragraphs below, the most common laboratory processes are described, as they are carried out on small amounts of materials.

MELTING POINT. It does not often occur that one has insufficient material for a melting point determination in the usual way, but when such is the case, the value of the melting point for identification can hardly be over-estimated, since so few other tests are possible with such a small quantity of substance.

The following are amongst the best methods which have been suggested for this purpose. They all depend upon the use of a so-called "hot stage." There are numerous makes upon the market, of various degrees of elaborateness, the best types being electrically heated. Leitz make a good model.

It is not difficult to devise a piece of apparatus which will efficiently replace these rather expensive varieties. A thin-walled, flat, and narrow bottle should be obtained; some scent bottles are suitable, if they have a wide neck, but the glass cells used for spectroscopic work on liquids are also good. The neck of the bottle is fitted with a rubber stopper, through which pass two small glass tubes, and an Anschutz thermometer. The tubes are of unequal length, one passing down to the bottom of the bottle, the other being flush with the cork, and they serve to introduce and carry away the heating medium, which is either water or glycerine, or a high boiling point ester.

The heating may be carried out in a variety of ways, but, in all cases, the liquid passes through the heating apparatus, enters by the long tube into the hot stage, leaves through the short tube, and is led off into the sink or a beaker. The temperature is regulated by the rate of the flow and the amount of heat applied.

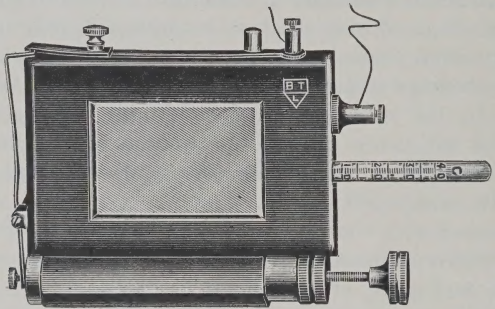


Fig. 72. Electrical Hot Stage (*Baird and Tetlock*)

One form of heater is a glass tube bent in the form of a spiral, of 2-in. diameter and ½-in. pitch, which is suspended in a tin cylinder; the lower tube of the spiral is bent upwards along the outside of the spiral, parallel with the axis. The heating is carried out by means of hot air from a Bunsen flame, and very steady regulation of the temperature can be obtained.

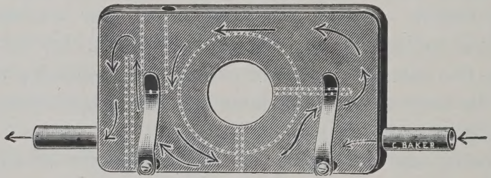


Fig. 73. Hot Stage (*Baker*)

A further apparatus, much simpler, though liable to some irregularity of temperature, is merely an ammonia flask containing the liquid. The heated liquid is drawn out from the side tube, from the bottom of the vessel.

The complete apparatus is used as follows: A speck of the substance is placed on a cover glass on the stage, and is focused through a 32-mm. objective. The temperature of the stage is slowly raised, and the apparent melting point noted. The actual melting point will be from 5° to 10° lower than the temperature recorded by the thermometer, but the exact amount to be allowed at any temperature is readily found by determining the apparent melting point of known substances. The stage is now allowed to cool, and the substance to solidify.

The next step is to place tiny specks of substances with known melting points, in a small circle round the unknown substance. The melting points are chosen to give a range of 3° or 4° on each side of the rough melting point already found. The stage is now slowly heated once more, and by observation under the microscope it is found which of the known substances melts at the same temperature as the unknown. This method, carefully carried out, gives results of considerable accuracy with microscopical particles. Draughts must be avoided, and it is wise to protect the lacquer work of the stage by means of a sheet of asbestos.

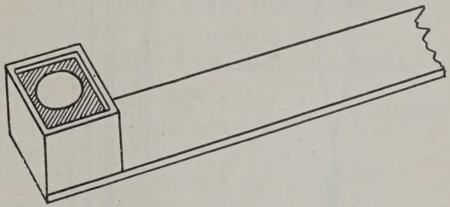


FIG. 74. COPPER HOT STAGE

A second method, apparently crude, but which gives excellent results, is to make a hot stage from a strip of copper 8 in. long, by brazing on to one end a small copper box, 1 in. square and 1 in. deep. This is half-filled with Wood's metal, or mercury; Wood's metal is preferable, because the apparatus can be put away when not in use, with the metal still in it. The speck of substance is placed on a No. 1 cover glass, which floats on the metal; it is focused as before, and the temperature gradually raised by heating the other end of the copper in a Bunsen flame. Thermometers are inserted in the metal. The copper strip is best supported on a thick layer of asbestos.

One method may be mentioned, which, in addition to being of interest, provides a means of obtaining the melting point of anisotropic substances, and when a good hot stage is used, the results are very accurate. These substances, when viewed under polarized light, become completely invisible the instant that the crystal structure is destroyed by melting.

A modification of the usual method of taking a melting point is as follows:

A substance in suspension in a fluid such as water or benzene, may be sucked into a capillary tube (as used for boiling points method 2), the end sealed, and this liquid evaporated by the device indicated in Fig. 75. The inner tube is a fine capillary thread only, but serves to evaporate the liquid quite rapidly combined with gentle warmth. The solvent is evaporated, leaving the substance deposited in a thin film on the wall of the capillary tube. The melting point is now taken as usual, the end point being observed through a magnifying glass or with a 4 in. objective in the microscope.

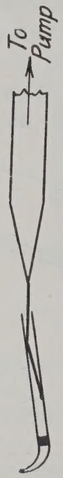


Fig. 75. Evaporation of a Drop of Liquid

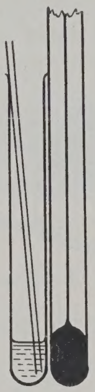


Fig. 76. Boiling Point Determination by the Capillary Tube Method

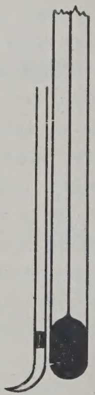


Fig. 77. Boiling Point Determination by the Melting Point Method

Boiling Point. The boiling point of a few drops of liquid may be determined to within a degree by the following method—

The liquid is placed in a test tube made from very thin walled glass tubing, $\frac{1}{4}$-in. by 2-in. size, which is fastened by a rubber band to a thermometer, in much the same style as when taking a melting point by the capillary tube method. A $2\frac{1}{2}$-in. length of fine capillary tubing, such as is used for melting point determinations, but unsealed at both ends, is inserted in the liquid. The whole apparatus is then placed in a beaker of water or glycerine, and the temperature gradually raised. At the boiling point of the liquid, bubbles will issue from the lower end of the capillary.

When only one drop of liquid is available, the test tube described may be narrowed at the lower end to $\frac{1}{3}$ in. or less. It often happens in this case that, at the boiling point, the bubbles are evolved so rapidly that the liquid is spurted out of the tube.

Very accurate results may be obtained by this convenient and rapid method.

A further method, which uses an even smaller quantity of liquid, makes a clever use of the fact that at the boiling point the vapour pressure of a liquid is equal to atmospheric pressure. A capillary tube is employed, as for a melting point estimation, with the difference that one end is drawn out to a very fine capillary thread. A drop of the liquid to be examined is drawn into the tube through the fine capillary, which is dipped in the liquid, and after removal of the tube, the drop is drawn a little way up the tube, so that a small air pocket is below it. The fine point of the tube is sealed in a flame and the capillary attached to a thermometer by one of the customary methods, the two being then immersed in a liquid of high boiling point. The rest of the procedure follows the lines of an ordinary melting point determination; at the boiling point the drop of liquid rises in the capillary tube to the height of the surface of the heating bath. The success of the method depends upon the size of the trapped air bubble.

Specific Gravity. A capillary pyknometer is made from a length of thin-walled glass tubing of fine bore, to the shape shown in the sketch. The end *A* is immersed in the liquid, which is drawn in past the mark *B* by suction at *C*. The level is adjusted exactly to *B* by applying a piece of blotting paper at *A*. With half a cubic centimetre of liquid, results sufficiently accurate for most purposes may be obtained.

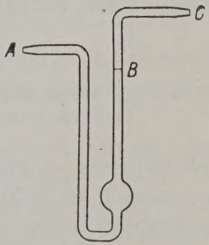


Fig. 78. Capillary Pyknometer

The calculations are exactly the same as when using an ordinary specific gravity bottle.

The specific gravity of a single drop of liquid may be obtained by placing it in one of the mixtures of liquids given under the section on gravity separation, with which it is immiscible, and varying the proportions until the drop does not rise or fall. The S.G. of the unknown liquid is then the same as that of the mixture employed. The results are only approximate.

Refractive Index. If a drop of Canada balsam be beaten up on a slide by means of a glass rod, a mass containing very many small air bubbles is obtained. When this is covered with a cover glass in the usual way for mounting objects for microscopical examination, we have in effect a substance of low refractive index (air), mounted in a medium of high refractive index (Canada balsam).

An experiment is easily made by focusing one of these bubbles under a 32-mm. objective, with the substage condenser as close to the slide as possible, and the diaphragm three-quarters closed. The position of the condenser is of importance because, by lowering it sufficiently far from the slide, it is possible to obtain results exactly the opposite of those to be now described.

The image of the air bubble will be seen to be surrounded by a few dark rings, and coloured fringes can usually be very easily seen. The distance apart of these rings, their general appearance, and their intensity depend on the relative refractive indices (*n*) of the substance and the mountant, their relative dispersive power, and the wavelength of the light employed.

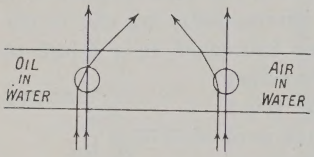


FIG. 79. REFRACTIVE INDEX DETERMINATION

It is obvious that such a bubble, or in fact any substance having a lower *n* than has the mountant, must tend to act like a concave lens; i.e. causing parallel rays passing through it to become divergent. Should these conditions be reversed, the medium having the lower *n*, as with oil bubbles in water, the opposite applies, and the substance will cause parallel rays to become convergent.

"n" of Substance less than "n" of Medium. The rays emerging from a spherical fragment of the substance, if originally parallel, will become divergent. In consequence, the appearance of the object, as seen when in correct focus, will apparently "expand," when the objective of the microscope is raised, throwing the substance out of focus. The object is surrounded by a bright ring of light, which is outside the one or more dark bands or contour rings, and as the objective is raised, this bright ring will be seen to move outwards, away from the centre of the substance.

If one now places a piece of stiff cardboard across the bottom of the condenser, in such a way as to shut off all light from entering the left half of the condenser, the right half of the field of view will be dark, whilst the left half will still be well illuminated. The light, however, is coming obliquely through the condenser, and this oblique pencil of light is made divergent by passing through the substance; the diagram sketches the path taken. It is not strictly accurate, but will demonstrate the effect. It is evident that there is a strong tendency for the light from the side of the substance farthest from the dark half of the field to be so much deflected that it will not enter the objective at all, whilst the side of the object nearest to the dark half of the field will deflect the light in such a way that it will all enter the objective.

That is, the object will appear dark on the side near to the light half of the field, and light on the opposite side.

"n" of Substance Greater than the "n" of Medium. Parallel rays passing through the substance will in this case tend to become convergent, and hence, upon raising the objective from the position of correct focus, the bright ring, which is in this case seen *within* the dark contour bands, will apparently contract and move towards the centre of the substance.

When illuminated with a pencil of oblique light, also, the tendency is for the light from that side of the substance nearest to the dark half of the field to be so much deflected that it will not enter the objective. Hence the opposite effect will be observed to the one already described, and the object will be dark on the side nearest to the dark half of the field, whilst it is bright on the side farthest from the dark half of the field.

The following convenient mnemonic rule is given by Emich: *Beim **h**eben des Tubus wandert die **h**elle Linie zum **h**öherbrechenden Medium.*

"n" of Substance Equals "n" of Medium. The black contour rings will be entirely absent. One would expect the substance to be invisible, and it is a fact that a good deal of difficulty is experienced in observing an object which is so mounted, but in practice the object usually has some foreign matter on its boundaries, and air bubbles or internal imperfections of structure. For the present, it is more important that the object will rarely have the same dispersive power as the medium. The eye normally adjusts itself during observation to the portion of the spectrum round about the green-yellow region, and it is on this that the refractive index is determined, since this light band is by far the most important for visual purposes. In addition to the probable difference in dispersive power, solids are found, generally speaking, to have a higher *n* for blue rays, and a lower *n* for red rays, than have liquids. The result is that usually the red rays emerging from the substance tend to be convergent, when the condition at the head of this paragraph is satisfied for the green-yellow wavelength. Therefore, in spite of the fact that there are no interference rings to be seen, the image is edged with blue on the inside, and with red on the outside. On raising the objective from the position of correct focus, the blue fringe concentrates on the centre of the substance, whilst the red fringe expands away from the centre.

Method of Finding "n" of a Solid. The solid is placed on the slide, and a drop of some liquid which is thought to have about the same refractive index is placed on it. The mount is then focused with a 32-mm. or 16-mm. objective, and the rings noted; the objective is raised out of focus, and the movement of the bright rings watched; finally, a piece of cardboard is interposed below the condenser as described, in order to obtain oblique lighting, and it is observed which side of the substance darkens. By means of these observations, which take only a few seconds, it is ascertained that the liquid is of higher or lower *n* than the solid. The liquid is blotted off with filter paper, washed by placing a drop of a liquid deemed nearer to the true *n* on the object and blotting it off again, then adding a second drop for the purpose of taking an observation. In this way, after a few trials, one may find a liquid of the same refractive index as the solid. With a little practice the *n* may be found easily to within ± 0·007–8.

Monochromatic light, obtained by the employment of a colour screen, may be employed if greater accuracy is required. The *n* thus found may be roughly converted to any given wavelength value by assuming that *n* increases by 0·001 for every 15λ for solids.

The following mixtures of liquids may be employed (Chamot)—

Acetone and chloroform	1·36–1·44
Chloroform and bromoform	1·44–1·58
Alpha monobrom naphthalene and bromoform	1·58–1·65
Alpha brom naphthalene and methylene iodide	1·65–1·74
Methylene iodide and either sulphur antimony iodide, or arsenious sulphide	1·74–1·96

In addition, the following common liquids may be used in many cases—

n	Substance
1·32	Methyl alcohol
1·36	Ethyl ether
1·37	Ethyl alcohol
1·40	Amyl alcohol (b.p. 132° C.)
1·44	Chloroform
1·46	Carbon tetrachloride
1·47	Glycerine
1·49	Benzene
1·53	Chlorbenzene
1·55	Nitrobenzene
1·57	o. toluidine
1·58	Bromoform
1·62	Carbon disulphide

It need only be added that the refractive index of a liquid, which is probably of more general analytical value than that for a solid, may be obtained in exactly the same way, by substituting solids of known *n* for the unknown substance already dealt with. The following substances are of value in this connection.

n	Substance
1·439	Sodium alum
1·450	Potassium alum
1·459	Ammonium alum
1·481	Potassium chrome alum
1·485	Ammonium iron alum
1·490	Potassium chloride
1·494	Rubidium chloride
1·502	$NaAc \cdot UO_2 Ac$
1·515	Sodium chlorate
1·544	Sodium chloride
1·553	Rubidium bromide
1·559	Potassium bromide
1·566	Strontium nitrate
1·571	Barium nitrate
1·640	Ammonium chloride
1·645	Caesium chloride
1·650	Rubidium iodide
1·657	Potassium stannochloride
1·667	Potassium iodide
1·698	Caesium bromide
1·700	Ammonium iodide
1·755	Arsenious oxide
1·788	Caesium iodide
2·071	Silver chloride

SOLUTION. The solubility or insolubility of a substance is often of great importance, especially when the solute is a reagent such as caustic soda. When the solubility is great, no appreciable difficulties are met with in deciding the fact, but when the substance is insoluble, or is a mixture which is only partially soluble, this is not the case.

Again, in microanalytical procedure, very often only small quantities of material are available, or perhaps only one drop of solution is required.

To determine the solubility or otherwise of the substance in a reagent such as HCl, the following method may be adopted. A minute fragment of the material is placed in a drop of water on a slide, and heated gently until a clear and saturated solution is obtained. On cooling this rapidly by placing the slide on a slab of lead or stone, minute crystals are usually obtained, unless the solubility in water is very great, when the drop will set solid, and the test cannot be applied. A drop of the reagent is added, when the crystals will disappear almost instantly, if soluble. Should the substance be insoluble in water, the test may be carried out by rubbing it into a thin cream with water, by means of a glass rod ground flat at one end. The reagent is then added as before. The test is most useful when the substance is fairly soluble hot, but is insoluble when cold.

To test the solubility in some solvent such as water, the substance is added to a drop of the solvent, rubbed into a cream, and if thought necessary, gentle heat applied. The drop is then allowed to stand for a moment and filtered. The filtrate is transferred to a clean slide and allowed to evaporate. A residue, of course, denotes solubility.

In order to obtain a drop of solution for the purpose of some test, a large drop of water, 1 cm. in diameter, is placed on a 3-in. by 1-in. slide, and gently warmed until it is steaming slightly. A small quantity of the substance is now placed in the drop, and the mixture further warmed. As soon as there seems no further signs of solution, the liquid is decanted off, or filtered.

A small Bunsen flame which is just non-luminous will be found quite suitable for heating purposes, although a small platinized glass jet is also good. The portion of the slide bearing the drop should be brought into contact with the tip of the flame perhaps half a dozen times, for a fraction of a second only, and the drop should only be allowed to steam. Boiling must be avoided, for, apart from its being unnecessary, the evaporation becomes very rapid, as the glass round the drop also becomes considerably heated; in addition, the boiling of small drops is always rather spluttery and explosive. Some workers recommend that the drop be placed in one corner of the slide, but provided that thin slides be employed, and the heating be carried out with reasonable care, there is no danger of the slide being cracked, even when the drop is in the centre.

It should be mentioned that for chemical tests, the most expensive microscope slides are the worst, for they are made of soft glass which is readily soluble in alkaline reagents. The harder glass cheap slides are better.

Needless to say, this method may only be used when the solvent has a reasonably high boiling point, and is non-inflammable. Low boiling-point solvents, which are usually also inflammable, must be treated in a 2-in. by ½-in. test tube, which is conveniently flattened at the end to a shape which will enable it to be stood upon the bench. If desired, a small CO_2 flask may be easily made from a short length of ⅜-in. glass tube. This form of flask has many advantages. Its area of evaporation is small, it may be corked up if required, it may be stood upon the bench with safety, and its neck is sufficiently short to allow of a drop being taken out by means of a glass rod, without any difficulty. The heating is best carried out by a bath of glycerine in a small beaker. Direct heat always results in bumping.

When the solution is deemed complete, the micro flask is held under the tap for a second, and wiped dry. Any residue in the flask is allowed to settle, and the clear liquid decanted or filtered off, if the nature of the solid permits.

In certain cases, when it is found necessary to extract a solid repeatedly with a solvent, a micro-Soxhlet may be employed.

It is constructed as follows: The outside flask is made from an ordinary ½-in. by 6-in. test tube, by softening the lower portion in a bunsen flame, and making three indentations about 1½ in. from the bottom, with the point of a small triangular file, or the point of a lead pencil. The substance is wrapped in a small piece of filter paper, which is twisted loosely into a bag, and is placed in the inner tube. This is made from a 3-in. length of ¼-in. glass tubing, which has been drawn out to an hour-glass shape, and cut in the middle of the narrow part to make each half 1½ in. long. One half is employed. The solvent is placed in the test tube to a depth of about 1 in. and is boiled by means of a glycerine bath; the vapour passes by the inner tube, is condensed by the micro-condenser, falls back on to the substance, percolates through, and extracts the soluble portion in the same way as does the usual large apparatus. A little practice will soon show the correct relative sizes of the two tubes.

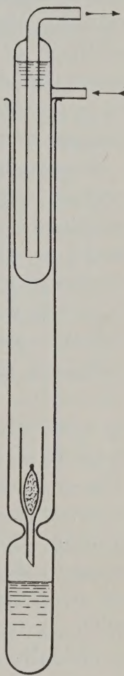


FIG. 80. MICRO-SOXHLET APPARATUS

The micro-condenser requires a little skill in glass working for its making. In brief, a side tube is fused into a ⅜-in. by 2½-in. test tube, ½ in. from the top; the top opening is then closed up slightly, and a second tube is fused into this in such a way that it goes almost to the bottom of the test tube. A better way is to use some ⅜-in. tubing of the same make as the side tube, since the two glasses will then combine more easily; in addition, the manipulation is somewhat simpler, since the bottom of the "test tube" is made last, according to the length of the inner tube. The condenser water goes in through the side tube and out at the top tube. When in use, it will be found sufficient simply to rest it inside the micro-Soxhlet by means of its side tube, without any supporting cork. This also reduces any liability of impurities being introduced.

EVAPORATION. The drop of solution or suspension is merely placed on a slide, and evaporated by gentle heat by holding it some distance away from the point of a small Bunsen flame. If any considerable residue is present, a further trial should be made in order to obtain, if possible, some characteristic crystals.

CRYSTALLIZATION. Owing to the high surface tension which obtains in a drop of liquid, there is a strong tendency for a drop of solution to become supersaturated in place of crystallizing. Again, when a drop is simply placed on the slide and allowed to evaporate the crystallization which may take place usually begins at the edges, and the crystals so obtained are usually of little value for identification purposes. Strong solutions, which are caused to evaporate rapidly, also give masses of ill-defined or deformed crystals. The addition of reagents which force back the dissociation, or which lower the solubility of the substance which it is desired to crystallize, have the same result unless a means can be found of introducing the reagent very slowly and steadily. (See Figs. 101 and 102.)

Successful crystallization in microscopical analysis requires a certain amount of practice and care. The best results will be obtained by using the type of slide which has a glass cell, $\frac{1}{8}$-in. deep and $\frac{1}{2}$-in. diameter, cemented on the surface. These cells hold three to four drops of the liquid. The solutions are often found to "creep" over the walls of the cell rather badly, unless some precaution be taken to stop this; smearing the rim with a little vaseline for water solutions, or with a little soap for many organic solvents, will prove successful. Water solutions may very conveniently be crystallized in a cell which is made by pressing a very thin roll of plasticine into a circle of $\frac{1}{2}$-in. diameter on an ordinary slide, to form a low wall. The crystallization may be stopped at any desired stage by pressing a cover glass on to the plasticine by means of some flat object, the mounts thus obtained being permanent for a few weeks. Such mounts should, however, be stored at a constant temperature, for an alternation of warmth during the day and cold during the night, with consequent partial solution and deposition of the substance, eventually may completely alter the original appearance.

Three or four drops of the liquid are placed in the cell and allowed to evaporate at the ordinary room temperature. After an hour, the centre of the cell will show a mere film of liquid, and crystallization will commence here, the crystals being very well formed and separate, and easy to examine under a 16-mm. objective. The shape and other characteristics having been noted, the liquid is allowed to evaporate to dryness, during three or four hours, and the general appearance of the crystal mass is noted. Many substances which give closely similar crystals at the first stage form completely unlike masses at the conclusion of the evaporation.

When the crystallization has as its object the purification of a few milligrams of substance, rather than the observation of crystal form, the solution should be prepared in the micro flask, and filtered through a micro funnel. Crystallization is carried out in a deep watch glass, and the crystals washed by decantation, or by filtration through the micro vacuum funnel, and air-dried, or dried in a desiccator. Substances which prove only slightly soluble even in the hot solvent are dissolved in 15 cc. of liquid, and after any necessary filtration the solution is allowed to cool in a small centrifuge tube. When the crystallization due to cooling is complete, the tube is centrifuged. The crystals usually form a solid plate at the point of the tube.

Crystallization may be effected in certain cases by saturating the water solution with the vapour of some liquid such as alcohol, in which the substance is insoluble. A drop of the solution is placed on a slide, which is allowed to rest on a watch glass containing the alcohol, ether, acetone, pyridine, etc., the whole being covered with the half of a Petri dish. After a few minutes, well-formed crystals will usually have been produced. This method may also be adapted with success to the crystallization of a few organic bases from alkaline solution by standing over conc. HCl, or in the crystallization from concentrated sulphuric acid by standing over water, or the precipitation of lead chloride from solutions of lead salts by standing over HCl.

The crystallization of fusible solids is carried out by placing a fragment of the solid on the slide (covered by a cover glass) and heating cautiously until melting takes place. The slide is placed on a metal plate, and the cover glass pressed down firmly until the mass has solidified. The crystal form is sensitive to the conditions of cooling. Chamot gives as an example monochloracetic acid, which gives three forms according as crystallization is induced by rapid cooling, by scratching the outside of the cover glass, or by seeding the edge with a crushed crystal.

Instructive preparations are obtained by crystallizing from a solution which on cooling sets to a jelly, e.g. gelatine water. Rhythmic crystallization often takes place.

SETTLING. One often meets with liquids containing a fine suspension which would be lost on a filter paper. When, as is the case with river water and so on, there are a few litres of liquid, a convenient method of obtaining the solid matter is to fill a Winchester quart with the water, and invert it into an evaporating dish which contains a few cubic centimetres of the liquid. The suspension settles down into the neck, and can be obtained by removing the bottle rapidly from the basin. The concentrated sediment may then be treated by centrifuging for complete separation, if required.

With 100 c.c. only, a conical separating funnel will be found best. The funnel should have the stem cut off to within a $\frac{1}{4}$ in. from the stopcock in order not to waste any sediment in the tube.

When only a few cubic centimetres of liquid are available, and it is not thought advisable to use the centrifuge, as, for example, when the suspension consists of delicate crystals, the device illustrated in Fig. 81 may be employed. It consists simply of a 3-in. length of $\frac{1}{2}$-in. diam. glass tubing, which has been drawn out at one end, and cut so as to have an orifice of $\frac{1}{8}$ in.

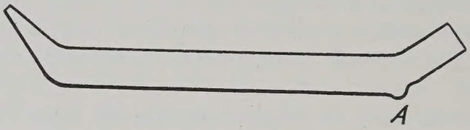


FIG. 82. SEPARATION TUBE

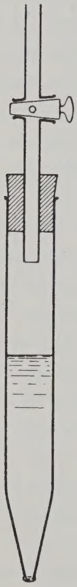
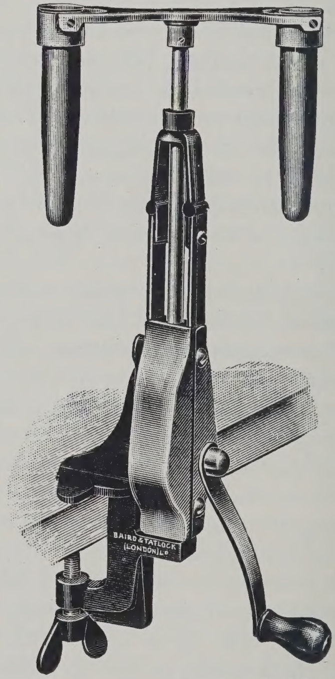


FIG. 81
SETTLING
TUBE



(*Baird and Tatlock*)
FIG. 83. CENTRIFUGE

The wide end is fitted with a cork through which a short tube fitted with a stopcock passes. The cork is removed, the lower end of the tube closed with the finger, the liquid is then poured into the tube, the cork replaced, and the stopcock closed. When the finger is removed from the end, a few drops of water run out, but the rest remains in the tube, which is placed in an upright position in a clamp, and allowed to stand until the sediment is collected in a small drop hanging from the end. The drop may be removed in a small watch glass, or on a microscope slide, by simply touching it, and the sediment will be largely contained therein. Very small quantities of suspension may be collected in this way in sufficient quantity for microscopical examination.

Centrifuging. The use of a centrifuge merely accelerates the process of settling, but in practice, the instrument is more often used as a rapid means of filtration, more especially in cases where settling could not be applied, as with urine, which rapidly putrifies, or when filtration would be impracticable, owing to the corrosive nature of the liquid to be treated; or, again, when precipitates such as barium sulphate must be dealt with, which are too finely divided to remain on filter paper, but can be readily concentrated by centrifuging.

Emulsions may also be broken very readily by this method, and, of course, there are a number of well-known analytical processes based on the fact, of which the estimation of fats in milk may be cited.

In general, centrifuging will be found an extremely valuable asset to the microscopist, when small quantities of precipitates are being examined.

There can be no doubt that the best forms of centrifuge are those which are electrically driven, though excellent work may be done with the small and much cheaper hand-driven types. A two-tube centrifuge, carrying 15 c.c. tubes, will be adequate for most purposes, the larger sizes being more applicable for work on water and emulsions.

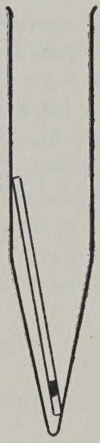


Fig. 84
Recovery of a Drop of Liquid

The centrifuge enables a convenient recovery to be made of the drop of liquid used for boiling point determinations by the second method given. The capillary tube is bent slightly, the fine tip broken off, and the tube placed in a wider tube (which contains the rest of the liquid), as shown in the sketch. A slight swing in the centrifuge causes the drop to be expelled from the capillary tube.

Gravity Separation. A well-known analytical process is the separation of coffee and chicory by shaking the roasted "coffee" with a liquid which consists of a mixture of two volumes of glycerine and one volume of water. Any coffee present floats to the surface, whilst the chicory sinks. The separation is effected by flooding the test tube with the glycerine-water mixture, so that the coffee particles float over into another receptacle.

This method finds very useful employment in micro technique. By the choice of suitable mixtures, two substances may be completely separated when no other method could be of value, either due to the nature of the material or the small amount available.

Textile sizes, for example, may be dissected by the use of a mixture of chloroform and bromoform of S.G. 1·8. Starch and flour float, whilst the inorganic constituents sink. Quite often even three and four fractions may be obtained and examined microscopically. It should be noted that sometimes a long time should be allowed for the settling, more especially when the S.G. of the two substances lie close together, but the separation is considerably hastened by the use of the centrifuge.

A further example may be cited. Acetate silk may be separated from viscose by means of a 40 per cent solution of potassium iodide in water. The viscose sinks, as do also wool and cotton. Cellulose acetate silk and true silk float.

The following pairs of liquids will be found useful in this connection. Care should be taken to select a medium in which the substance is not soluble.

	S.G.
Chloroform	1·526
Bromoform	2·884
o. toluidine	1·003
m. toluidine	1·998
Alcohol	0·800
Carbon tetrachloride	1·595
Water	1·000
Glycerine	1·270
Water	1·000
Pyridine	1·965

SIFTING. When dealing with powders which consist of a mixture of particles which differ fairly considerably in size, a separation may often be effected by the use of a sieve of appropriate mesh, as estimated by the particle sizes seen under the microscope. Even when a complete separation cannot be in this way effected, one component may usually be concentrated and its examination thus made easier.

For general purposes the substance may be placed in a watch glass, finely woven silk cloth which is tightly stretched over it being used as a sieve, but the best results are obtained by the use of sieves of known gauge, which have been checked microscopically.

Information concerning sieve standards may be obtained from the following sources: *Journal of the Society of Chemistry and Industry*, 7th May, 1926, 128 T.; U.S. Standard Sieve Series, Bureau of Standards, Letter Circular No. L.C. 74; B.S.I. Table for Wire Screens; Institute of Mining and Metallurgy, Standard Table for Laboratory Screens.

FILTRATION. Several very ingenious methods have been devised by various workers for the filtration of one drop of liquid, and, indeed, filtration is one of the most successful operations in microanalysis.

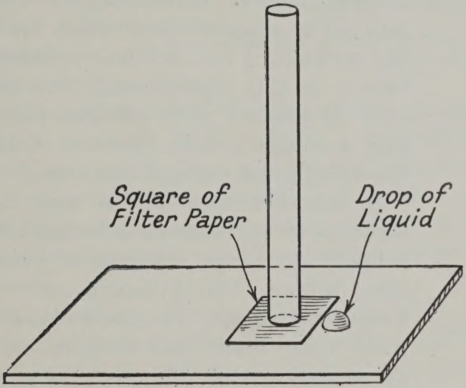


FIG. 85. FILTRATION OF A DROP OF LIQUID

The method of filtration is very simple. A small piece of filter paper, about $\frac{1}{2}$ cm. square, is placed on a microscope slide, near to the drop which is to be filtered. A 6-in. length of glass tubing which has been ground flat at one end, is pressed evenly on the filter paper, and this is then moved along the slide until the drop is being absorbed. The liquid is completely drawn into the tube by suction, and retained there by stopping the top end with the tongue, until the drop is safely transferred to another slide, or another part of the same slide. The precipitate, or sediment, remains at the edge of the filter paper.

This method may also be applied in certain cases for the separation of an oil from a drop of water, by using wet filter paper.

The main points to be watched are that too much suction is not applied, and that the ground end of the tube is pressed evenly and squarely on the filter paper.

The process outlined may be modified for the purpose of dealing with quantities of liquid up to 5 or 10 c.c., by making use of a 10 c.c. pipette, which has been cut off 1 in. above the bulb, and 3 in. below. A small roll of filter paper or asbestos is packed into the end of the long limb, and the short end is connected to the filter pump. The liquid to be filtered may then be contained in a small test tube.

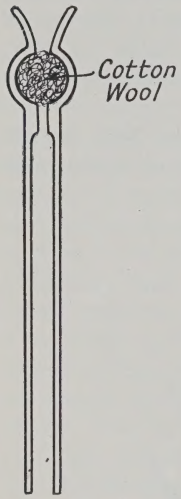


Fig. 86. Micro Funnel

A micro funnel, for dealing with somewhat larger amounts of precipitate, is made as follows: A piece of $\frac{1}{8}$-in. diameter glass tubing is fused into a $\frac{1}{2}$-in. diameter tube which has been tapered to, perhaps, $\frac{1}{8}$ in. and has a small glass bulb blown on the tapered end. For use, the small bulb is packed with asbestos fibre, and fitted into a Buchner flask. Suction must be used to draw the liquid through the funnel.

A micro-vacuum funnel may also be constructed on the lines of Schwinger's original idea, from a length of $\frac{1}{2}$-in. glass tubing. This is drawn out to a cone, taking care that the walls are made fairly thick, and the cone cut off at such a point that the end is a $\frac{1}{4}$ in. in diameter. A piece of narrow-bore (capillary) tubing of the same external diameter is ground flat, and the end of the cone is similarly treated. A small spiral of platinum wire is made, of the same diameter, and a circle of hard filter paper cut to this size. A length of rubber tubing, $\frac{3}{4}$ in. long, is cut to serve as a connection, and placed on the ground end of the small bore tube. The platinum coil is pushed down to the glass, together with the filter paper, and the cone is then connected up so that the platinum and the filter paper are tightly held between the two ground glass surfaces. The apparatus will stand a full vacuum quite easily, and almost negligible quantities of precipitates may be collected and washed with ease, even when colloidal in character.

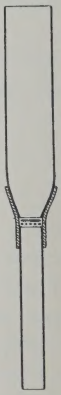


Fig. 87 Schwinger's Vacuum Filtration Funnel

Fig. 88 shows a form of filtering tube given by Emich; it is made by slightly thickening the walls of a glass tube at the end, and about 1 cm. from the end, the intervening bore being packed with cotton wool or asbestos fibre.

Pipettes. For washing precipitates, a 10-c.c. pipette is the best;

the tip should be almost closed by means of a bunsen flame, so that a very fine jet of water only is emitted. The tip should not be drawn out to obtain the fine bore, as it is then very fragile. This pipette may also be employed to wash precipitates on a microscope slide, when only a very small quantity of water is needed.

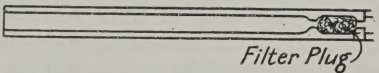


Fig. 88. Emich's Filter Tube

A fine pipette may be made from thermometer tubing by drawing it out to the shape indicated. This may be calibrated in graduations, each of which is equivalent to 0·05 c.c.

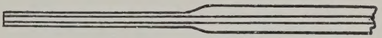
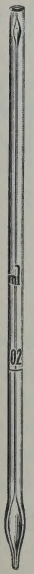


Fig. 89. Pipette from Thermometer Tubing



(*Baird and Tatlock*)

Fig. 90 0·2 c.c. Pipette

Such a pipette may be used for the purpose of adding reagents to drops on the slide. A drop of the reagent is placed in a watch glass, and some of this is transferred by means of a pipette to the testing drop, thus preventing the possibility of the reagent being contaminated. Capillary tubes are also useful for this purpose, being thrown away after use.

Decantation. The decantation of a single drop of liquid is accomplished as follows: The slide is gently inclined at an angle soon learned by experience, and the drop of water is led along a narrow channel by means of a platinum wire to a place 1 cm. away, on the slide. By now inclining the slide a little more the water will flow gently from one place to the other, leaving the solid matter behind. The connecting channel is cut by means of a piece of stiff filter paper, leaving the liquid and solid separate, to be dealt with as required. The slide must be very clean and free from grease.

Distillation. The separation of a few drops of liquid of sufficiently high boiling point, from a solid with which it is mixed, or which is held in solution, is very simply accomplished. The solution is boiled in a crucible of appropriate size, and the vapour collected on the underside of a watch glass containing cold water, which is used in place of the lid of the crucible. When the distillation is complete, the water is soaked up from the watch glass with a piece of blotting paper, and the liquid transferred to a microscope slide for a test to be made, or otherwise as desired.

The separation of two liquids mixed together is usually impracticable with less than 1 cub. cm. of liquid, and even then only when the boiling points are not very close together. The apparatus is quite simple to make. The micro-distilling flask is made from a length of $\frac{3}{8}$-in. glass tubing, by fusing a side tube in, which is inclined upwards, not, as is usual, with a slight downward trend, and which is then, about $\frac{1}{2}$ in. from the joint, bent downwards at the usual angle. This has two advantages, for any slight splashing or bumping which may take place does not give rise to the danger of some of the undistilled liquid being introduced into the distillate, and also, when a water bath is being used for heating the flask, water vapour condensing on the side tube runs back into the water bath, instead of trickling down into the distillate. Should this still tend to happen, a rubber ring may be placed on the side tube just below the bend, or a ring chalked round with one of the wax pencils used for writing on glass ware in the laboratory. The flask is completed by blowing a bulb on one end of the wide tube about 2 in. from the joint, and on the opposite side of the joint, cutting the tube about $\frac{1}{2}$ in. away, to hold the cork.

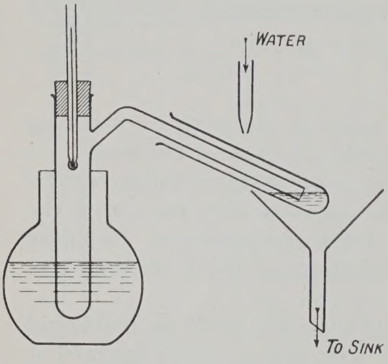


FIG. 91. FRACTIONAL DISTILLATION

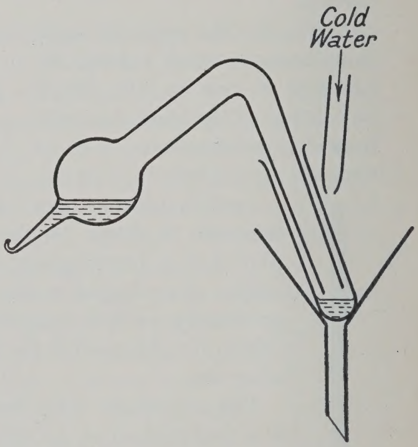


FIG. 92. FRACTIONAL DISTILLATION

When very small quantities of liquid only are available, the apparatus shown in Fig. 92 may be employed. It may be made in a few moments from a piece of glass tubing, and serves very well if the boiling point of the fractions is taken by the second method. The fractions may be withdrawn as they collect, one drop at a time, by the capillary tubes. The fine capillary on the end of the bulb is formed by touching the red-hot end with a piece of hot glass rod, and instantly withdrawing it. The tip is broken off, the liquid sucked into the bulb, and the tip of the capillary sealed.

The apparatus is very convenient and satisfactory, and should always be used when only a few drops require handling.

An even simpler distilling flask may be made in the form shown in Fig. 93.

The heating should always be carried out by means of some bath. Glycerine will be found generally convenient, as it has a high temperature range, and even at high temperatures, the smell of its decomposition products is not disagreeable, as only small volumes are employed. It is readily washed off under the tap, and the flasks are not oiled up. If an oil be preferred, then well-blown cotton-seed oil of m.p. 60° C. will be found useful, as it solidifies to a powder which may be dusted off quite easily. The plasticizers used in the cellulose lacquer industry, e.g., tricresyl phosphate (b.p. 400° C.) may be used with advantage.

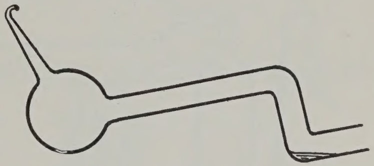


FIG. 93. SIMPLE APPARATUS FOR FRACTIONAL DISTILLATION

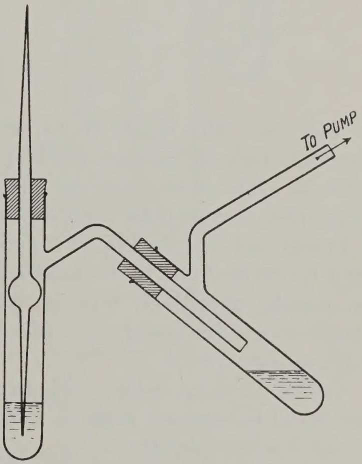


FIG. 94. VACUUM DISTILLATION

The flask should be supported in the CO_2 flask containing the glycerine by means of a cork which has V-shaped cuts filed in the edges. The flask should not be supported on a wire gauze, but should be heated directly with the smallest flame which will give the required temperature for distillation.

The receiving flask is either one of the small 2½-in. by ⅜ in. test tubes, or a second micro-distillation flask. The latter is preferable for it may be supported by means of its side tube. Condensation is most conveniently brought about by directing a small stream of water on to the outside of the receiver, the water which runs off the end being collected in a funnel, and led off into the sink. Fractions of four drops are collected, and their boiling point determined separately, as elsewhere described.

Vacuum distillation may be carried out by slightly modifying this arrangement, as shown in Fig. 94. A fine capillary tube is used to prevent bumping, as is customary, and the wide portion of the capillary tube is conveniently blown into a bulb in order to prevent any splashing into the side tube.

Pregl devised a piece of apparatus for vacuum distillation which is made out of a test tube, but which, however, only permits of one fraction being collected. A modification of his apparatus is shown in Fig. 95. The liquid to be distilled is drawn in through the capillary tube by suction at the pump, and the end of the capillary tube is then drawn out to the required fineness. The receiving apparatus is cooled by water as already described.

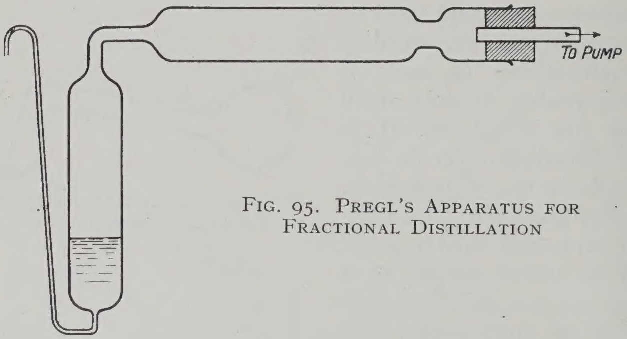


Fig. 95. Pregl's Apparatus for Fractional Distillation

Fractional distillation of a couple of cubic centimetres of liquid may be carried out by making the neck of the micro-distilling flask previously described, 8 in. or 9 in. long. The 6 in. of neck thus obtained will be found quite efficient. The neck should be wrapped with asbestos wool.

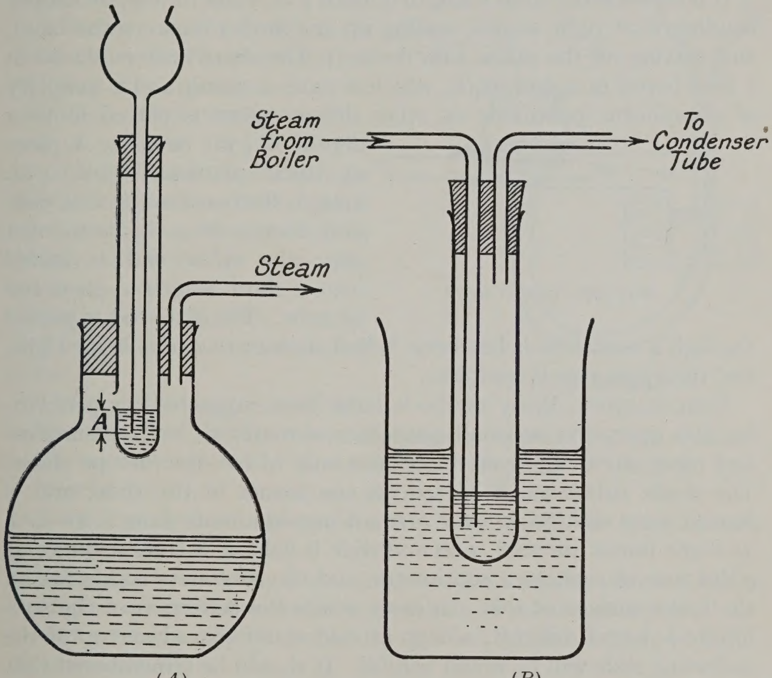
Steam Distillation. This process, troublesome and lengthy on the usual laboratory scale, is comparatively simple and convenient in microscopic routine.

The distilling apparatus (Fig. 96 *B*) containing water and the drops of substance to be distilled is heated in a glycerine bath to slightly over 100° C. The steam boiler (Fig. 96 *A*) is fitted with a device which prevents any possibility of the liquid which is being distilled, being sucked back if the flame is removed from under the boiler. All the usual steam distillation processes may be carried out conveniently and rapidly.

Separation. The separation of two immiscible liquids is a very difficult matter when only a few drops are available, and the author knows of no good method.

If only one or two liquids are required, as, for example, an oil to be freed from water, the end of a strip of filter paper may be wet with water, and applied to the drop, when the water is gradually soaked up by the paper, together with a little of the oil.

If the required liquid is the lighter of the two, the mixture may be placed in a $2\frac{1}{2}$-in. by $\frac{3}{8}$ in. test tube which has had one side of the lip bent inwards for $\frac{1}{8}$ in. With a little care, the lighter liquid may be largely poured over the lip into another receptacle, such as a small watch glass, leaving the heavier liquid behind in the tube, together with some of the lighter one.



(*A*) (*B*)

Fig. 96. Steam Distillation

(*A*) Boiler and safety valve (*B*) Distillation tube

A must be less than the dip of the steam tube in the liquid in 96 *B*.

If the required liquid is the more dense of the two, then the small apparatus described under Settling may be used, the cork and stopcock being discarded. It is really a modified form of separating funnel. The ordinary form of separating funnel is too large, the stopcock is very liable to introduce impurities from the lubricant employed, and the stem also results in too much of the liquid being lost, when only small volumes are being used.

Desiccation. For the most part, no special desiccator is necessary, but when a solid or liquid must be thoroughly dried very rapidly, then the form of micro desiccator now to be described is very efficient.

It is constructed from a length of hard $\frac{3}{8}$-in. glass tubing, by simply bending it at right angles, sealing up one limb 1 in. from the bend, and cutting off the other 2 in. distant. The short limb is placed in a hole bored in a 2-in. cork, which acts as a stand, and a quantity of phosphorus pentoxide or other drying agent is placed in to a depth of $\frac{1}{2}$ in. or $\frac{3}{4}$ in. A piece of thick platinum wire, 1 in. long, is flattened out at one end, and beaten into the form of a cup; the other end is sealed into a short length of glass rod or tube. The glass rod is passed through a cork which has been boiled in wax to make it air-tight, and the apparatus is complete.

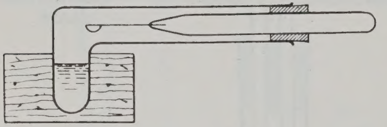


Fig. 97. Desiccator

Sublimation. Many methods have been suggested for performing this operation on small quantities of material, but the simplest and most often employed makes use only of two microscope slides. The crude substance is placed on one corner of the slide, and is heated very cautiously over a small non-luminous flame. As soon as slight fumes are seen, a second slide is held over the substance at a distance of perhaps a centimetre, and the sublimate condenses on the lower surface of this. In cases where the collection of the sublimate is found difficult, a drop of cold water placed on top of the collecting slide will be found helpful. It should be remembered that many substances condense in the liquid form, and only crystallize after standing for some time.

A complete separation of two substances, only one of which will sublime, may be effected by carrying out the sublimation in a thin-walled glass tube of $\frac{1}{4}$-in. internal diameter, and 5 in. long, which is sealed at one end. The substance is inserted through a roll of glazed paper so that none of it touches the sides of the tube. After the paper has been withdrawn, the tube is immersed to a depth of 2 in., or less, depending upon the quantity of material available, in a glycerine bath which is kept at the appropriate temperature until it is considered that the sublimation is complete. This may take half an hour. The tube is then taken out of the heating bath, the glycerine wiped off, and the tube cut into two parts in such a way that the substances are separated. A cover of asbestos over the glycerine bath is advisable in order to keep the upper part of the tube cool.

With care, two substances which both sublime, though at different temperatures, may be separated in this way, and their crystalline form examined.

A further method, which is often conveniently employed, is to place the substance to be examined in a small crucible, and in place of the lid of the crucible, to employ a microscope slide; direct heat is used, from a small flame, as the method is mainly of advantage when the substance sublimes only at a rather high temperature. A large part of the sublimable substance is lost.

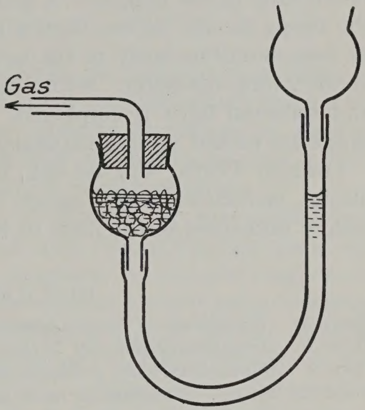


FIG. 98. GAS GENERATOR

GASES. Gases may be evolved in the first tube of the steam distillation apparatus, and washed in the second tube. If it is not deemed necessary to wash the gas, it may be produced in a micro-distillation flask.

A small gas generator may be made from two thistle funnel heads on familiar lines (Fig. 98.)

If the gas is to be used as a reagent, the solution which is to be treated is placed in a small tube which is used exactly like the receiving flask in distillation, or it may be led into a beaker containing the solution.

An excellent method of treating a drop of liquid with a gaseous reagent is to place the chemicals forming the gas in a ring cell and place over this a cover glass carrying on the underside a drop or film of the solution to be tested.

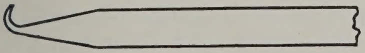


FIG. 99. ANILIDE TUBE

ANILIDE TUBES. These very convenient reaction tubes are made from glass tubing by drawing them out to the sketched shape. In the preparation of organic derivatives, the tube is heated in a bath until the reaction is complete. The tube is then taken out, wiped clean, and the capillary broken, when the contents may be blown out, with almost no waste, into a small watch glass for further treatment. Alternatively, the contents may be observed by mounting the tube in dilute glycerine. Observation of the contents of an unmounted round tube is very difficult.

COLOUR REACTIONS. A drop of the reaction mixture which is suspected to be coloured is placed upon a slide side by side with a blank drop of the reagent. A mere pin-point drop is necessary, and the drops should be less than a millimetre apart, so that they can be seen simultaneously in the field of a 32 mm. objective, which is about 2 mm. diameter. White cardboard is placed under the slide, and reflected light is employed. The test is more sensitive if the drops are picked up in a capillary tube, as described on page 28.

THREAD TESTS. Viscose silk, impregnated with reagents such as litmus, provides a useful means of carrying out colour tests. It is simply immersed in the drop to be tested.

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CHAPTER VII

INORGANIC MICROANALYSIS

In the more usual analytical methods, an endeavour is made to separate the elements by means of group reagents which precipitate completely certain elements under definite conditions of acidity, alkalinity, and so forth. The reagents are chosen because of the low solubility of the compounds which they precipitate.

The identification of inorganic compounds by micro-analytical methods is, however, usually based upon the appearance under the microscope, i.e. the crystalline structure, of certain compounds of the elements. In the majority of cases the usual group reagents such as ammonia, sulphuretted hydrogen, and so on, are more or less useless because of the amorphous or colloidal character of the precipitates which they give. Again, a compound of very low solubility is in the majority of cases precipitated either in an amorphous condition, or in very minute crystals, and hence those reagents which give very insoluble precipitates are also not employed in microanalysis.

It should be mentioned, however, that Dr. E. C. Grey has worked out a method of analysis based upon the usual analytical tables, making use largely of the colours of the precipitates produced with the three reagents $(NH_4)_2CO_3$, KI, and $(NH_4)_2S$.

The most usual microanalytical reagents owe their value to their property of forming double salts, the solubility of which, though low, is not so low, and hence the salts so rapid of formation, as to make it difficult to obtain good crystals. For example, in the usual analytical methods, barium sulphate is a better precipitate than ammonium magnesium phosphate, because it is so much more insoluble; in microanalysis, the reverse is the case.

A further difference in the routine of the two methods is found in the fact that these double salts are usually formed independently of other elements which may be present. Thus, precipitation of a mixture of copper and cadmium by sulphuretted hydrogen results in a mixture which must be separated; in microanalysis, the precipitate obtained with potassium mercuric thiocyanate would give immediate and definite visual evidence of the presence of both elements.

A third distinction is that microchemical methods are much more rapid than the others. In testing a material for tin, a drop of the solution may be evaporated to dryness several times with nitric acid, extracted with water, to remove other substances, the residue dissolved in aqua regia, the excess acid evaporated off, the stannic chloride dissolved in water, and the presence of tin conclusively established by the addition of caesium chloride, in five minutes.

A further and most important advantage is the great economy in the use of the material to be analysed. A very complete analytical determination may be carried out on an amount of material which would hardly suffice for one test on the test-tube scale of operation.

Finally, there is the increased sensitiveness of the results to be considered. Certain of the tests, such as those for copper and cobalt, leave nothing to be desired in their certainty, and in the indications given of the merest traces of the elements.

Owing to the peculiar nature of the tests, it is perhaps advisable to deal in detail with the reagents employed, and the results obtained, rather than with the individual elements and their reactions.

In microchemical analysis the metals are grouped very closely in accordance with the periodic table, and this arrangement should be constantly borne in mind when interpreting results. When a test is to be made for any particular element, an account will be found under the reagent heading of the special conditions desirable, and of the reactions of other elements which may interfere or cause confusion. Most metals, of course, give some indication of their presence or absence with the other group reagents.

Precipitation methods. (*a*) A drop of the substance or its solution is placed on the slide, in one corner, and a deeper drop of the reagent is placed, perhaps, 1 cm. distant, in the nearest corner. The reagent is led into the drop along the short edge of the slide, by means of a platinum wire, or a thin glass rod, and should be allowed to flow slowly through the narrow channel thus formed. The drops should not be allowed to coalesce completely at first. The insoluble compound formed will lie in the centre of the solution, and in the majority of cases will be crystalline. If it is thought that a little more reagent would be advantageous, the slide may be tilted to allow a little more to flow. If too much reagent is present, it may be partially removed by inclining the slide in the opposite direction. The crystals may be observed through a 16-mm. objective, using a ×10 eyepiece. The method is generally applicable, and there are very few reactions which may not be carried out in this way, provided care be taken. In certain cases the crystals are too small to be distinctly observed through a 16-mm. objective; the 4-mm. objective should then be used, the drop being covered with a cover glass in this instance. When no crystals are immediately produced

TABLE I

KEY TO INORGANIC MICROCHEMICAL REACTIONS AND TESTS.

	Thiocyanate	Caesium Chloride	Caesium Sulphate	Ammonium Fluoride	Potassium Nitrite	Triple Nitrite	Double Phosphate	Bismuth Sulphate	Platinic Chloride	Potassium Iodide	Hydrochloric Acid	Sulphuric Acid	Oxalic Acid	Potassium Chromate	Ammonium Chromate	Sodium Bicarbonate	Nitro-prusside	Sodium Bismuthate	Dimethyl Glyoxime	Uranyl Acetate	Ammonium Hydrate	Perchloric Acid	Pot. Ferrocyanide	Zinc	Separation of Metal
Ag . .	X	—	—	—	—	—	—	—	—	X	X	X	—	—	X	—	—	—	—	—	—	—	—	X	—
Pb . .	—	—	—	—	—	X	—	—	—	X	X	X	—	—	X	—	—	—	—	—	—	—	—	X	—
Hg . .	—	—	—	—	—	—	—	—	—	X	X	X	—	—	X	—	O	—	—	—	O	—	—	—	X
Cu . .	X	X	—	—	—	X	—	—	—	X	—	—	—	—	—	—	O	—	—	—	—	—	X	X	—
Cd . .	X	—	—	—	—	—	—	—	—	—	—	—	O	—	—	—	O	—	—	—	—	—	—	—	—
Bi . .	—	X	—	—	—	—	—	O	—	—	—	X	—	—	X	—	—	—	—	—	O	—	—	O	—
As . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	—	—
Sb . .	—	X	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	O	—
Sn . .	—	X	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	X	—
Fe . .	O	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	—	O	—	X	—	—
Cr . .	—	—	O	O	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	—	—
Al . .	—	—	X	O	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	—	—
Zn . .	X	—	—	—	—	—	—	—	—	—	—	—	O	—	—	X	O	—	—	—	—	—	—	—	—
Mn . .	O	—	—	—	—	—	O	—	—	—	—	—	O	O	—	—	O	O	—	—	—	—	—	—	—
Ni . .	O	—	—	—	—	X	O	—	—	—	—	—	—	—	—	—	O	—	X	—	—	—	—	O	—
Co . .	X	—	—	—	X	X	O	—	—	—	—	—	—	—	—	—	O	—	—	—	—	—	—	O	—
Ca . .	—	—	—	—	—	—	—	—	—	—	—	X	X	—	O	O	—	—	—	—	—	—	O	—	—
Ba . .	—	—	—	—	—	—	—	—	—	—	—	X	X	—	X	O	—	—	—	—	—	—	O	—	—
Sr . .	—	—	—	—	—	—	—	—	—	—	—	X	X	—	—	O	—	—	—	—	—	—	—	—	—
Mg . .	—	—	—	—	—	—	X	—	—	—	—	—	—	—	—	—	—	—	—	O	—	—	O	—	—
K . .	—	—	—	—	—	—	—	O	X	—	—	—	—	—	—	—	—	—	—	O	—	O	—	—	—
Na . .	—	—	—	—	—	—	—	O	O	—	—	—	—	—	—	—	—	—	—	X	—	—	—	—	—
NH_4 .	—	—	—	—	—	—	X	O	X	—	—	—	—	—	—	—	—	—	—	O	—	O	—	—	—

X = Of first importance. *O* = Reaction given, but not so valuable.

scratching the slide with a glass rod often causes precipitation, as these small drops of liquid readily become super-saturated. When this fails, the drop must be allowed to evaporate. Heat should never be employed unless it is known to be advantageous.

The type of crystal may, and usually does, vary considerably according as the precipitation takes place in a hot concentrated solution, or a cold dilute one. The two photographs of crystals of caesium alum will illustrate this very forcibly. (Figs. 101 and 102.)

(*b*) It should be remembered that many reactions require a considerable excess of one or other of the components. Method (*a*) gives usually an excess of the metal. By causing the unknown solution to flow into the reagent drop, the reverse state of affairs is brought about.

(*c*) In many cases the precipitated compound is soluble in excess of one component. Unless conditions are correct, no precipitate may be obtained at all. Should this be considered possible, a small fragment of the solid reagent may be added to the drop of solution. The reagent slowly dissolves, and the effect is to produce a zone round the reagent in which the reagent is in great excess, a more distant zone where the conditions are correct for precipitation, and a further zone—the remainder of the drop—in which the reagent is not sufficiently concentrated to produce a precipitate. Thus, there will always be some of the insoluble compound formed.

(*d*) When the compound produced by addition of the reagent is rather soluble, and owing to the dilution of the solution no precipitate is observed, the procedure described under crystallization on page 94 is to be followed.

(*e*) In some cases precipitation may be best effected by allowing a volatile substance to saturate the reagent with its vapour. This may be accomplished in many ways, one of the most satisfactory being to place the drop of the substance 1 cm. away from the reagent drop and to cover them both with a watch glass. When the reagent is not very volatile, the test drop may be treated on the underside of a slide, which rests on a crucible containing the reagent.

(*f*) Volatile reagents may usually be allowed to react as described under (*e*), but as they may be produced in quantity, the test is best made as follows: The reagent is placed in a microdistilling flask, and a drop of the liquid substance or solution is picked up by a short length of $\frac{1}{8}$-in. glass tube, by simply touching it on the slide. The tubing, which should have been previously passed through a suitable cork, is now placed in the micro flask, and when the reaction is complete, the drop containing the crystalline precipitate is re-transferred to a slide for examination.

(*g*) The method given under (*f*) may also be employed for the various colour tests such as the sodium nitro-prusside test for sulphuretted hydrogen, and comparative drops of untreated solution may be placed on the slide for comparison.

The double Thiocyanate reaction. This is a most important group test, which gives very valuable indications of the presence of Zn, Cu, Cd, Co, whilst Ag, Pb, Au, Mn, Ni, Pb, and Fe also give precipitates which may prove at times to be of importance.

A drop of the reagent is mixed with a drop of the solution in water of the substance. When the solution of the substance is very dilute, a dilute reagent must be used and the mixture, if necessary, allowed to crystallize slightly. (For reagent, see page 377.)

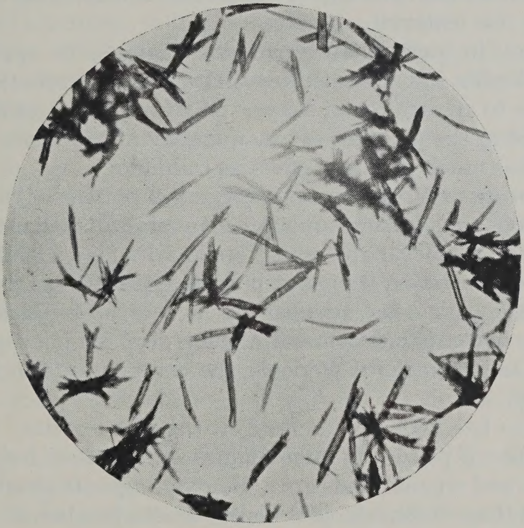

FIG. 100. COBALT-MERCURY DOUBLE THIOCYANATE

ZINC. A precipitate is formed immediately, which is seen to be in the form of small white feathers, in groups of crosses or branches. Though white by reflected light, it often happens that they are black by transmitted light. The precipitate has the formula $Zn(CNS)_2$, $Hg(CNS)_2$.

Copper in minute traces merely tints the feathers brown or mauve, the colour deepening to black with increase in the amount of copper present. When much copper is present, however, the feathery structure is not much in evidence, and black ball-like masses are obtained. The feathery form is, nevertheless, always produced to some extent before these irregular clumps of crystals are seen.

Cobalt in minute traces has the effect of colouring the feathers blue, but as the amount increases, there is a gradual transition from the form of the pure zinc salt to that of the pure cobalt salt. If cobalt is present in large amounts, zinc cannot be detected by this method.

When both cobalt and copper are present, the zinc crystals take on a most unusual and characteristic appearance.

A large amount of magnesium has no other effect than that it slows down the crystallization of the zinc salt. Aluminium also in large quantities has little effect, except that it may cause the crystals to become less feathery.

Cadmium, in quite small quantities, changes the appearance of the zinc double salt considerably. The feather formation usually gives place to an arrowhead shape. Small quantities of zinc cannot be detected in the presence of any quantity of cadmium.

Nickel has much the same effect as cadmium.

Iron, ferrous or ferric, has no effect unless present in large quantities. Ferrous salts in large amounts give a result similar to the pure copper double thiocyanate, whilst ferric salts give a pink colour to the solution, in traces; if present in such a quantity as to colour the solution deeply, a very peculiar result will be obtained, for the feathers at first separate as usual, though deep red in colour, instead of white, and then quite suddenly the crystalline form is changed completely.

Lead also has little or no effect on the test for zinc. Silver will only interfere if present in large quantities, and then not always.

Colloids and organic acids are a disadvantage. It should be noted that in all these tests, colloidal bodies should be absent.

Copper. The solution should be only faintly acid, and must, of course, not be alkaline; the best condition is neutral. It should not be too concentrated, although the crystals take some time to come down in dilute solution. The double salt appears as a radiating mass of soft-looking needles, yellow-green in colour. If only traces of copper are present, a little zinc sulphate should be added to the solution before adding the reagent, in order to obtain the lavender tinted zinc crystals, which are characteristic of copper.

Cobalt. The test drop should be preferably neutral, or slightly acid. The double salt is in this case deep blue-black in colour, and comes down in orthorhombic prisms which are usually not very well developed, and often take some time to form. Nickel may be present in large quantities without interfering, as it gives no crystals under the usual analytical conditions.

Cadmium. The double salt usually separates in long prisms, many of which appear singly. Organic salts of cadmium give poor results. Copper colours the crystal in the same way as with zinc, and if much copper is present, the copper double salt is first thrown down, later a mixture of the pure cadmium type with this being seen. Iron may colour the crystals brown. Magnesium and aluminium have no effect; lead and silver do not usually interfere. Zinc in any quantity usually prevents the prism formation, whilst in small quantities a peculiar kind of crystal first appears which is later followed by the usual cadmium salt.

Nickel. The reaction has no value as a test for nickel. In very concentrated solutions, yellow discs may be formed.

Manganese. The crystals may be very similar to those of cadmium, especially in very concentrated solutions, or solutions which are acid with sulphuric acid.

Iron. Ferrous salts in concentrated solutions give crystals similar to those of copper.

Silver. White amorphous precipitate, which later crystallizes to a form similar to cadmium. As might be expected, the crystals are much smaller.

Caesium chloride double salts. This expensive reagent forms rather insoluble salts with many elements, and hence may produce a large number of crystal forms from a complex mixture. Double chlorides are given with Mg, Pb, Hg, Cu, Bi, Cd, Mn, Ni, Co, Zn; no double salts are formed with Al, Fe, Cr, Ca, Sr, Ba, K, Na, Li.

The reagent is chiefly of value for Sn, Pb, Sb, Hg; Ag and Hg may give amorphous precipitates.

Tin. If tin be suspected, a test drop should be evaporated to dryness repeatedly with nitric acid, to form metastannic acid. The residue is extracted repeatedly with dilute nitric acid, and is then dissolved in aqua regia, evaporated to remove excess of acid, and the stannic chloride thus obtained dissolved in water. The operation only takes a few minutes when performed very thoroughly, which saves much doubt later. The double chloride obtained with caesium chloride takes the form of small regular colourless cubes, or sometimes octahedra, which are very refractive. Ammonia and sodium yield more soluble double chlorides than does caesium, and should, therefore, be absent.

Antimony. Caesium chloride gives with antimony salts hexagonal plates which show lines like spokes of a wheel radiating from the centre to the angles. The conditions are rather difficult to control, and better results may often be obtained by adding a little potassium iodide. The hydrochloric acid should be present only in sufficient quantity to avoid the formation of basic salts of antimony, and when potassium iodide is added, it should be introduced as a small crystal at the opposite side of the drop to the caesium salt.

BISMUTH. The double chloride is very similar to that of antimony. The same conditions apply for the formation, and nitric and sulphuric acids should be absent if typical crystals are to be obtained.

COPPER. Yellow, rectangular plates, or sometimes red needles, are obtained, the colour deepening from yellow to red as the amount of copper present increases. The red needles are similar to those obtained with antimony when potassium iodide has been added, but are, however, very characteristic.

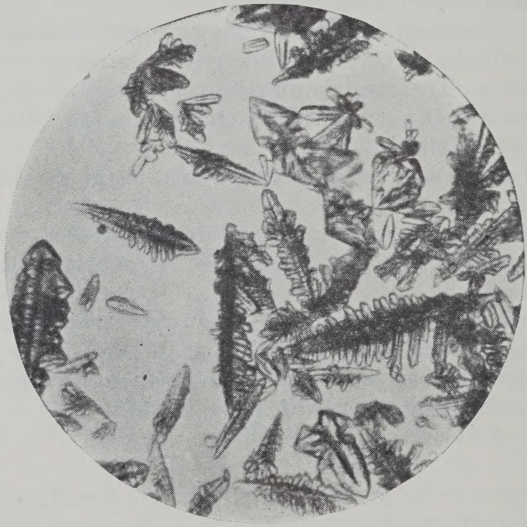

FIG. 101. CAESIUM ALUM, FROM A HOT, CONCENTRATED SOLUTION

Caesium sulphate double salts. This reagent readily forms, with a large number of metals, alums which are much less soluble than the corresponding potassium compounds. Though all the many possible alums are isomorphous, they are all difficult to crystallize, except the aluminium and chromium ones, and it is in this connection that the reagent is of value.

It is best to precipitate the test drop of solution with ammonia, wash the precipitate, and dissolve it in just sufficient dilute sulphuric acid. Much free sulphuric acid is very detrimental to the successful formation of crystals, and colloidal substances must be absent. In addition, dilute solutions are rather to be avoided, since they must be evaporated in order to obtain crystals at the ordinary temperature, and in this way a rather stable condition of supersaturation is easily obtained. Too concentrated solutions, on the other hand, result in the muddled network of badly-formed crystals.

As already indicated, the test is chiefly useful for aluminium and chromium, and is, in fact, the best test available for aluminium. Chromium requires a much more concentrated solution than does aluminium, and the crystals are a peculiar lilac shade. The pseudo alums may come down, but almost always form prisms and needles, which are thus easily distinguished from the true alums.

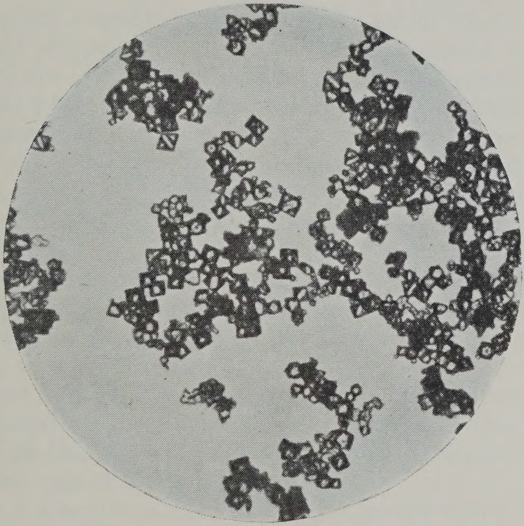

FIG. 102. CAESIUM ALUM, FROM A COLD, DILUTE SOLUTION

Ammonium fluoride double salts. It should be remembered that ammonium fluoride etches glass, and a slide which has been coated with a solution of celluloid in ether should be used. (See test for acids, page 138.)

ALUMINIUM. Neutral or faintly-acid solutions are employed, and the best method of working is to add a small crystal of the reagent, rather than a drop of solution. As this slowly dissolves, a ring of small crystals will make its appearance round the crystal of reagent in the form of small octahedra.

Chromium, Titanium, Iron, and Vanadium. These also give this reaction, though with difficulty, and the test cannot be regarded as successful from an analytical point of view.

Potassium nitrite double salts. A neutral drop of the solution of salt is mixed with the reagent, warmed slightly, and then acidified with acetic acid.

Cobalt. An extremely insoluble salt is produced, which appears amorphous, though it really consists of very small cubes, which are yellow by reflected light, though opaque to transmitted light. The precipitate should always for safety's sake be separated, decomposed by means of hydrochloric acid, and the cobalt confirmed by some other reaction such as the double thiocyanate.

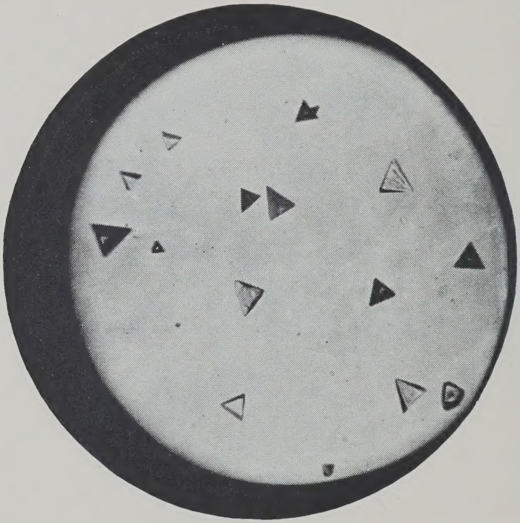

Fig. 103. Sodium Uranyl Acetate

Uranyl acetate double salts. Magnesium and the alkali metals give useful reactions with this reagent. No free acid should be present, and carbonates or hydroxides should be converted to acetate or chloride.

The calcium group is liable to cause trouble, and should be removed by sulphuric acid. Phosphates, if present, require an excess of the reagent to be used, and the liquid should be filtered off from the precipitated uranium phosphate and allowed to evaporate.

Sodium. Small pale yellow isotropic tetrahedra, black by transmitted light. If much potassium is present, it should be removed by adding an excess of perchloric acid, evaporating on a platinum foil, and extracting the residue with alcohol.

Potassium. Long needles or fine prisms, which are much more soluble than the sodium salt. *Ammonium, rubidium,* and *caesium* all give very similar crystals.

Magnesium. Large monoclinic crystals which may vary considerably in form.

Triple nitrite reaction. The triple nitrite in question is formed by the three metals—potassium, copper, and lead. The lead may be replaced in this salt by nickel or cobalt, and the copper by nickel, under the usual analytical conditions. The salt is somewhat soluble in excess of potassium nitrite, but some excess must be added to complete the precipitation. The test drop should be neutral, or only slightly acid with acetic acid, and free mineral acids must be absent, for which reason the presence of a little sodium acetate is advisable. The triple nitrite may be recrystallized by adding a drop of water containing a trace of acetic acid, and a little potassium nitrite, and boiling. Needless to add, strong oxidizing agents must be absent.

The test may be carried out as a reaction for lead, nickel, or cobalt, by adding copper acetate and potassium nitrite, or conversely, by adding lead acetate in place of copper acetate, a test for copper and nickel may be made.

To the test drop fragments of sodium acetate and of copper acetate are added, and the drop is well stirred with a platinum wire until solution is effected. A fragment of potassium nitrite is then added, and the slide placed under the microscope.

It is not too easy to obtain really good results with this reaction. Emich gives the following details: One drop each of 0·1 per cent lead acetate and 0·1 per cent copper acetate are mixed with the solution, warmed gently, cooled, and a drop of 50 per cent acetic acid (saturated with ammonium acetate and potassium nitrite) added to the mixture. Very little of the reagent is required. The crystals are rarely larger than about 10 mu.

Lead. Tiny squares, plates, or cubes of $K_2PbCu(NO_2)_6$ are formed, which are opaque to transmitted light but brown by reflected light.

Nickel. The triple nitrite is light brown by transmitted light.

Cobalt. The cubes in this case are very small, often merely appearing like a chocolate powder except under very high magnification.

The reverse test may be made for copper by adding lead acetate in place of the copper acetate to the test drop.

Copper. See Lead above.

NICKEL. Gives rise to light yellow-brown squares or cubes, which are never black.

The double phosphate reaction. This reaction is well known from its employment in the gravimetric estimation of magnesium, and may be represented by the equation

$$NH_4Cl+MgCl_2+Na_2HPO_4+NaOH=NH_4MgPO_4+3NaCl+H_2O.$$

It is employed in micro-analysis for the recognition of magnesium, ammonium, and manganese. Nickel and cobalt may also be detected by this means. Arsenates form a double ammonium magnesium arsenate, which is isomorphous with the phosphate.

AMMONIUM. Small fragments of sodium phosphate and magnesium sulphate are added to the test drop and well stirred to solution. Solutions of these salts may, of course, be employed, if desired ; when the solutions are rather concentrated this is the better method. The crystals may come down immediately, but usually a drop of dilute caustic soda must be added. The crystals separate in stars and crosses if the solution is concentrated, and also the crystals in this case are rather small, but later they develop into groups of rectangular prisms. It is necessary to take care that the phosphate and magnesium are not in excess, or they will be precipitated together in a form which is rather similar to the double phosphate.

Very many metals are precipitated as phosphates. Amongst these are Li, Ca, Ba, Sr, Mg, Al, Fe, Cr, Zn, Mn, Ni, Co, Pb, Cu, Ag. Of these, Li, Fe, Mn, Co, Ni, give salts isomorphous with the magnesium salts. It is therefore wise to distil off the ammonia from the precipitate which is thought to be the double phosphate, and collect the gas in a drop of water.

MAGNESIUM. The usual gravimetric method is followed quite closely. A crystal of citric acid is added to the test drop, followed by an excess of ammonia. The drop is evaporated to dryness, and taken up with a little warm, dilute ammonia. It will be found best to decant the liquid off from any insoluble residue. A small piece of sodium phosphate is added, and the crystallization proceeded with.

It is necessary to have sufficient ammonia present to form the compound, and also to ensure the slight alkalinity of the solution. Phosphates should first be removed.

MANGANESE. Ammonium chloride is added to the test drop, together with a slight excess of ammonia, and the mixture is then warmed gently. If any precipitate is produced, the drop should be filtered before proceeding further. A small crystal or drop of sodium phosphate is now added to the warm solution. The crystals are very similar to those given by magnesium, but are a little longer, and are turned brown by hydrogen peroxide.

NICKEL AND COBALT. The crystals given are isomorphous with the Mg salt, but the cobalt crystals are turned brown by hydrogen peroxide.

ARSENATES. A trace of ammonium chloride should be added, together with a little ammonia. The addition of magnesium chloride or sulphate now causes the precipitation of $NH_4MgAsO_4\ 6H_2O$, which is isomorphous with the double phosphate.

Bismuth sulphate double salts. This reaction is chiefly of importance as a test for bismuth, though it is also valuable for the recognition of sodium. This test is rather difficult to carry out successfully.

SODIUM. The salt is converted into the sulphate by evaporation to dryness once or twice with concentrated sulphuric acid. The sulphate is dissolved in water, slightly acidified with nitric acid, and a little bismuth sulphate added. The drop is warmed, and allowed to cool, when crystals of $3Na_2SO_4 2Bi_2(SO_4)_3$ are formed, as thin rods and prisms, with rounded ends, forming clusters and crosses. The crystals have parallel extinction. The calcium group should be removed, and free mineral acid should be present only in traces. It should be noted that fine greyish clusters, or thick prisms or plates, are not an indication of sodium.

POTASSIUM. Hexagonal plates and stars are formed.

AMMONIUM. The double salt is similar to the K salt.

BISMUTH. The method is similar to that for sodium, sodium sulphate being added in place of bismuth sulphate.

ARSENIC, ANTIMONY, and TIN do not give crystals. LEAD is preferably removed, since, although usually only a granular precipitate is formed, crystals may sometimes be obtained.

Double salts with platinic chloride. The reagent employed is really chloroplatinic acid, H_2PtCl_6, which may extract potassium from the glass of the bottle after long standing.

POTASSIUM. A neutral or very slightly acid (HCl) solution should be used. Very well-developed deep-yellow octahedra are formed, which may be so flat as to appear like hexagonal plates. If the solution be too concentrated, only small crystals are obtained. The salt is insoluble in alcohol.

AMMONIUM. Forms crystals exactly like those of potassium. When testing for potash, it is therefore advisable to remove any possible ammonia.

SODIUM. The double salt comes down as rather soluble not very well-developed triclinic prisms, which polarize well, and are soluble in quite strong alcohol.

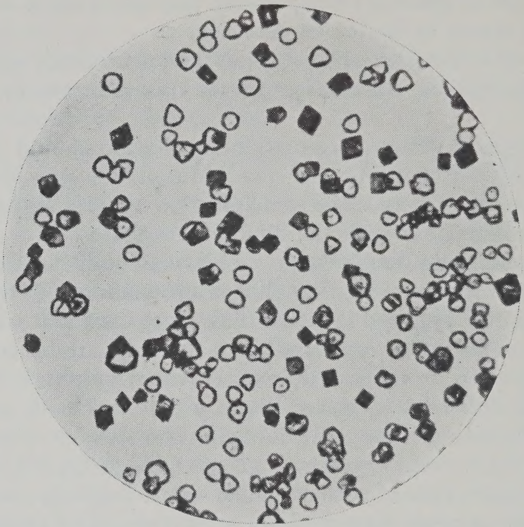

Fig. 104. Potassium Platinochloride

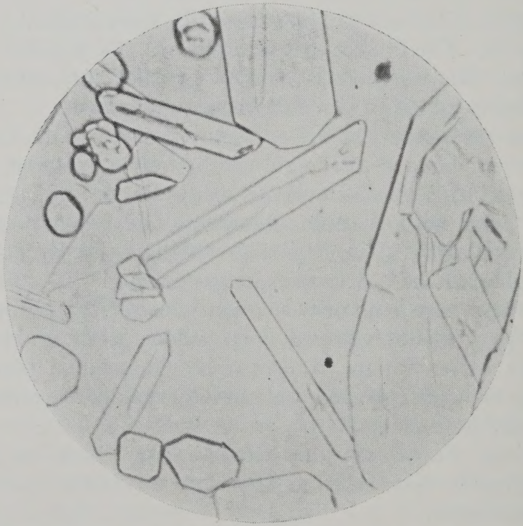

Fig. 105. Sodium Platinochloride

Potassium iodide reaction. A small drop of the reagent is added to a moderately concentrated solution of the substance. Excess should be avoided, for the precipitates are, in certain cases, soluble in the reagent, forming double salts with potassium iodide. These may be decomposed by adding an excess of water.

The metals which give precipitates of analytical value are Pb, Hg, Ag (Cu).

LEAD. The solution should be very slightly acid with nitric acid, because ill-formed basic iodides are precipitated from neutral solutions. Lead iodide is precipitated in hexagonal plates which glisten in reflected light. Very fine crystals are easily obtained by crystallization from hot water. If a little HCl is added during the crystallization, the hexagonal lead iodide is not obtained, needles of almost white PbICl being formed instead.

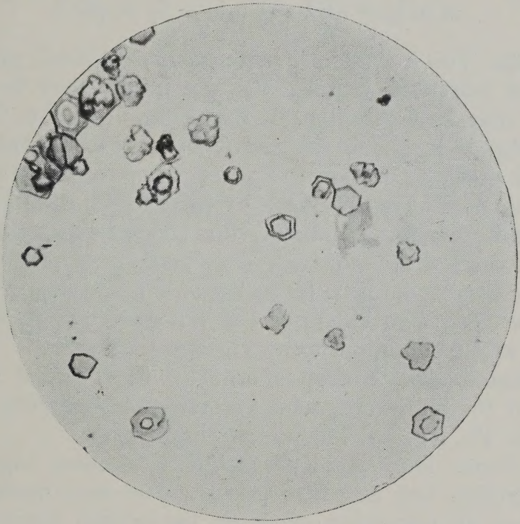

FIG. 106. LEAD IODIDE

Antimony and bismuth give double iodides which are easily formed. If, however, these two metals are both present with lead, a chocolate, sandy precipitate is obtained, quite unlike the double iodides given by these elements individually. This is partially decomposed on boiling with water, a certain amount of lead iodide being formed. The presence of a small amount of Bi alone, causes the lead iodide crystals to be much deeper and redder in shade, whilst the angles of the hexagons become less distinct.

SILVER. A yellowish mass is formed, usually amorphous, soluble in hot dilute nitric acid, and in hot water.

MERCURY. Mercurous salts give an amorphous precipitate, unless the solution is slightly acid with nitric acid; a little mercuric iodide is formed in this case, due to oxidation, a precipitate being obtained of red rhombic crystals.

COPPER. Both cupric and cuprous salts give a white granular precipitate of cuprous iodide. Iodine is separated at the same time with cupric salts, according to the equation—

$$2CuCl_2 + 4KI = Cu_2I_2 + I_2 + 4KCl.$$

Ammonia. This is a useful group reagent, but rarely gives definite results, for individual metals. The precipitate may be soluble in excess, or may be crystalline. It may contain Al, Fe, Cr, Mg (Sn, Bi, Sb, Hg).[1] It should be tested by repeated oxidation with nitric acid, and evaporation to dryness to convert any Sn into the metastannic acid. The residue is extracted with dilute nitric acid, and any solid remaining undissolved is treated with aqua regia and tested by means of CsCl for tin and antimony. Some of the precipitate should also be oxidized by means of boiling nitric acid containing a little potassium chlorate, any manganese thus forming the peroxide, whilst chromium forms chromic acid.

Hydrochloric acid. A drop of dilute HCl is added to a water solution of the substance, which is preferably slightly acid with nitric acid. Pb, Hg, Ag, Th, give precipitates.

LEAD. Forms long white characteristic needles, together with feathery crosses and dendritic prisms. The concentration of the solution has an important effect on the crystal form, and more characteristic crystals are obtained from fairly concentrated solutions. When the alkali metals are present in any quantity, double chlorides may be formed in dilute solutions. An excess of the reagent is advisable, and even then separation is never complete. The lead chloride is easily soluble in hot water, but the crystals obtained on cooling take the form of hexagonal prisms.

THALLIUM. Thallous salts give star-like clusters, which bear no resemblance to the lead salt. The difference is still more marked on crystallization from hot water, for the chloride comes down in cubes.

SILVER. AgCl forms an amorphous mass immediately. The precipitate should be washed with hot water slightly acid with nitric, which removes any lead or thallium. It should then be dissolved in cold ammonia, and the solution as soon as possible decanted off from any residue. On standing, very small cubes and plates are formed. Traces of silver in presence of mercury are difficult to detect.

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[1] The brackets indicate that reactions are given but are of lesser importance.

MERCURY. Mercuric salts, of course, give no precipitate. Mercurous salts form an amorphous mass which may, however, exhibit thin needles from very dilute solutions.

Sulphuric acid. Characteristic crystals are given by Ca, Sr, Ba, Hg, Bi, Ag.

A drop of the reagent is run into a drop of the solution to be tested.

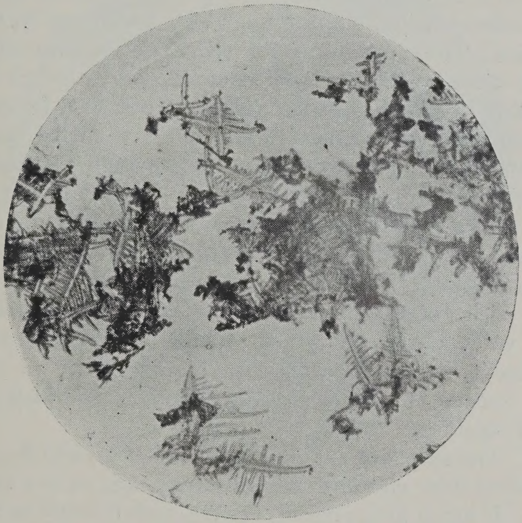

FIG. 107. LEAD CHLORIDE

Matters are simplified, when testing for the calcium group, if Pb, Hg, and Ag are first removed by HCl.

CALCIUM. Precipitation of the sulphate does not always occur immediately, and it may be necessary to allow the liquid to evaporate down at the room temperature; especially is this the case when an excess of strong mineral acids is present. By exposing the drop to alcohol vapour, the crystallization may be hastened.

The crystals formed are very characteristic, and the reagent may also be used for this reason when testing for calcium in plant structures. The slender needles are usually produced in the form of clusters or sheaves on first appearance, whilst subsequently larger swallow-tailed crystals, arrow-head shaped, are seen.

Other metals present do not seem to alter the crystal form. The sulphates of Sr, Bi, Hg′, may, under certain circumstances, be confused with Ca. In case of doubt, the liquid is decanted off, and a drop of ammonium carbonate solution is placed on the precipitate, which slowly dissolves; the solution deposits calcium carbonate in very small rhombic crystals. Fe, Al, and Cr, if present as chlorides, interfere very seriously, and should be removed by boiling in a large drop of water containing a little ammonium acetate, when they are precipitated as basic acetates. Borates also interfere.

STRONTIUM. A sandy precipitate is obtained, which very occasionally takes on a crystalline form. The precipitate may be recrystallized from concentrated sulphuric acid by breathing on to the hot acid solution. The appearance of the crystals thus obtained is difficult to describe, but is very characteristic, though lead may give a similar result. Strontium sulphate may also be crystallized from concentrated hydrochloric acid, when it forms thin-pointed prisms and sheaves. The operation is rather difficult, since crystallization sets in very rapidly on cooling. It is necessary to take a large drop of HCl and decant it from the residue immediately it reaches the boiling point. The test is important because barium sulphate is insoluble in HCl. Calcium should first be removed by extraction with hot water, or the strontium crystals will be of no analytical value. Lead and silver should be removed, but if lead only is present, its sulphate may be removed from the mixed sulphates by means of caustic potash solution, in which strontium sulphate is insoluble.

BARIUM. The sulphate is amorphous, and for identification must be crystallized from concentrated sulphuric acid as described under strontium. If much Sr is present, the precipitate must be repeatedly extracted with concentrated HCl; any lead sulphate should be similarly removed by means of KOH. The crystals obtained form crosses which usually have two arms much longer than the others.

MERCURY. Mercurous salts give fine needles at first, which are very similar to Ca but they are almost black by transmitted light, and after some time grow into thick prisms quite different from $CaSO_4$.

BISMUTH. The sulphate also is similar to that of Ca, but the sheaf-like form is absent, the needles are usually larger, the prisms have not often truncated ends, and curved hair-like forms are also present.

LEAD. Is precipitated as a powder, usually amorphous, but sometimes as orthorhombic crystals.

SILVER. Forms rhombs and prisms of quite characteristic appearance, colourless, and highly refractive.

Oxalic acid. The chief importance of this reagent lies in its salts with Ba, Sr, and Ca, though Zn and Cd also give useful crystals. It should be noted that the crystals from hot solutions may be completely different from those produced cold, and that free mineral acids are undesirable.

CALCIUM. The test drop should be neutral or slightly alkaline. The oxalate forms small square plates and octahedra with bundles of needle crystals. Free mineral acids change the form and make it much more soluble.

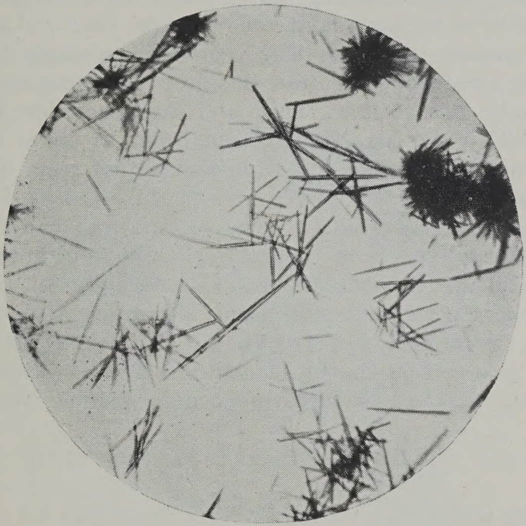

FIG. 108. BARIUM OXALATE

STRONTIUM. The oxalate is almost exactly like that of Ca, though the crystals are somewhat larger, and more rosettes are to be seen. The crystals should always be washed with water, and dilute sulphuric acid added to them, in order to make sure that none of the characteristic crystals of calcium sulphate are produced.

BARIUM. The oxalate is quite different from the Ca salt. It usually takes the form of large bunches of branched needles. The solution must be neutral, and hence it is advisable to add a little sodium acetate, though it must be remembered that the addition may allow magnesium oxalate to be thrown down. Sr and Ca interfere, and hence a little dilute HNO_3 should be added before adding the oxalate. This, though still allowing the oxalates of these two metals to crystallize, slows down the speed very considerably. After standing for some time, the liquor is decanted off from any crystals, sodium acetate added, and upon allowing to evaporate the barium salt comes down. If desired, of course, the mother liquor may be evaporated somewhat before adding the sodium acetate, when the Ba salt comes down immediately. Borates interfere. The chlorides of Fe, Cr, and Al also interfere, since they give double oxalates of peculiar appearance; their preparation is thus described by Chamot: "To the test drop containing barium, add ferric chloride in sufficient quantity to impart a faint yellow colour, then add a fragment of sodium acetate, and stir. The yellow colour should now have changed to a reddish tint. Into the drop thus prepared cause a drop of oxalic acid to flow. Tufts and sheaves of fine hairs soon appear, which grow longer, and soon begin to curve in a most peculiar manner. Calcium and strontium do not seem to interfere with the formation of this double oxalate of Ba and Fe." It should be added that the conditions are not very easy to control.

ZINC. Zinc oxalate is in general very similar in appearance to Ca salt, and hence, if testing for zinc, Ca and Sr should be removed. Cd also makes the test uncertain, whilst Mg causes plates of a double oxalate to be formed. Pb, Ag, Cu, Co, Ni, Fe, Al, Mn, Cr also interfere. As a test for Zn this reagent is not to be recommended.

CADMIUM. This gives long monoclinic prisms with oblique ends.

MANGANESE. Similar to cadmium; the ends of the prisms are dissimilar.

Perchloric acid. This reagent is chiefly employed for the separation of potassium from other substances. It forms colourless well-formed crystals of various kinds with potassium, caesium, rubidium, and thallium, in order of decreasing solubility, whilst good crystals are given by ammonia.

Potassium chromate. Potassium chromate is employed in place of ammonium dichromate, the general group reagent, because manganese bichromate is soluble. It is preferable to add a little of the solid reagent rather than solution.

MANGANESE. Gives bundles of yellow-brown needles from neutral solutions. Very weak nitric acid solutions cause the crystals to be thick prisms; stronger solutions slow down the precipitation greatly. The test is of no value if any other metal forming an insoluble chromate is present.

Sodium bismuthate. This reagent is employed as a test for manganese, though it is difficult to carry out successfully. Chamot describes the test as follows: "A nitric acid solution of the substances is evaporated down to dryness and redissolved in dilute nitric acid. Sodium bismuthate is added slowly in small quantities, and the precipitate of manganese dioxide which is formed is very exactly dissolved in sodium thiosulphate. A drop of nitric acid is added to the milky drop thus produced, and sodium bismuthate is again added very slowly and in small portions. Permanganate is formed, giving a pink coloration. The test is completed by adding rubidium chloride and perchloric acid. The rubidium perchlorate which crystallizes out, absorbs the permanganate, giving purple crystals."

Dimethyl glyoxime. NICKEL. This reagent provides the most sensitive test for nickel at our disposal. The presence of cobalt and copper in large amounts masks the reaction, and these metals should in this case be removed, the cobalt as nitrite and the copper by means of metallic zinc. Zinc and silver do not interfere.

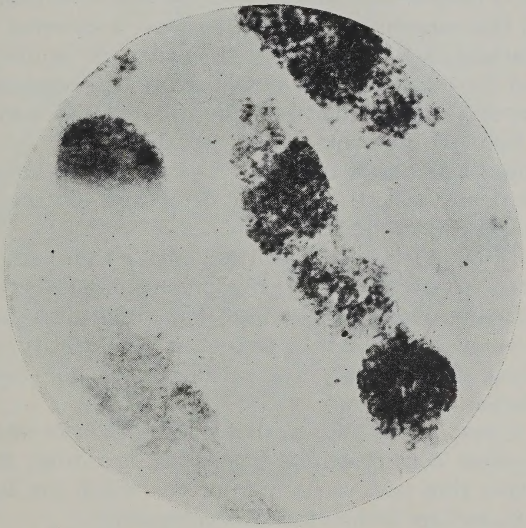

FIG. 109. NICKEL DIMETHYL GLYOXIME

The test drop is treated with ammonia in excess, and filtered off from any insoluble hydroxides. The filtered drop is allowed to flow into a saturated fresh solution of the reagent. Nickel gives a magenta precipitate which is amorphous at first, though later a tangle of fine needles is often produced.

No other metal than nickel gives this precipitate or anything like it; traces of iron remaining in the filtered drop may, however, cause a red coloration.

Potassium ferrocyanide reaction. The test drop should be slightly acid (acetic), and the presence of a little ammonium chloride is advisable. Indications are obtained of Ca, Sr, Ba, Pb, Fe, Zn, (Cu, Hg).

IRON. Dark blue precipitate or coloration is obtained, soluble in alkalis but insoluble in acids.

COPPER. Gives an amorphous brown precipitate. From an ammoniacal solution white crystals of $Cu_2Fe(CN)_6$, $2NH_3$ appear, which become red by the addition of acetic acid.

CALCIUM. Forms small square plates.

STRONTIUM. Gives no crystals.

BARIUM. Forms large yellowish rhombs, which are rather like the crystals of the reagent; they have, however, a different behaviour under polarized light. The reagent cannot be employed when mixtures of the alkaline-earth metals are being examined.

MANGANESE. Ferrocyanide is not precipitated from acid or neutral solutions, but only from ammoniacal test drops.

Ammonium bichromate reaction. A small piece of the solid reagent is in this case best employed. Reactions of value are given by Ag, Pb, Hg, Bi, Ca, Sr, Ba.

SILVER. From a weak acid solution (nitric), thin dark red plates are deposited. If insufficient acid be present, needles are obtained. The salt may be recrystallized from hot dilute nitric acid. Lead should be removed, since if it be present in any quantity, only small, yellow prisms are obtained. The calcium group does not interfere in acid solution.

LEAD. A weakly acid solution should be used; from cold solutions, a bright yellow non-crystalline precipitate is thrown down. Hot solutions give thin long monoclinic prisms which are insoluble in caustic soda, and are seldom found in groups.

MERCURY. The mercurous salts give a variety of crystal forms from dilute nitric acid solution, depending on the conditions. A dark red sandy precipitate, which develops into dark red bundles of crystals, is usually formed at the beginning (silver gives crystals which only rarely form bundles or crosses). These crystals are insoluble in caustic soda. Later on during the crystallization, some yellow crystals are usually formed.

BISMUTH. From dilute nitric acid solution, irregular and often poorly developed prisms, orange in colour.

CALCIUM GROUP. The test drop should not have any free mineral acid present, but should be slightly acid with acetic acid. From this solution, only barium is precipitated, in the form of small rods or yellow globular groups, mostly amorphous. The solution is decanted off, and neutralized with ammonia. On standing, the strontium salt settles out in dumb-bell like groups. Calcium gives no precipitate, either neutral or alkaline.

The bicarbonate reaction. Sodium bicarbonate is used as a reagent, a saturated solution being employed. Zn, Ca, Sr, Ba, give useful reactions.

Zinc. It is very important to have a considerable excess of the bicarbonate present, hence a large drop must be employed. The test drop is to be neutral or very slightly acid. An amorphous precipitate is at first obtained which soon changes into unique crystals, which take the form of tiny triangles and tetrahedra, with occasional clusters.

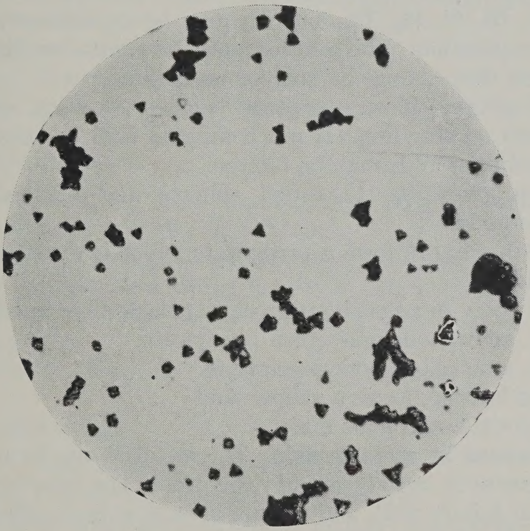

Fig. 110. Zinc Bicarbonate

Ca, Sr, and Ba, must first be removed by sulphuric acid. Mg, Ca, Pb, Fe, Mn, Ni, Co, also interfere, and zinc must first be extracted by oxidation with hydrogen peroxide, precipitation with ammonia, and extraction with fairly concentrated caustic soda; on acidifying the extract drop with acetic acid, zinc is thrown down. Cadmium may be removed by precipitation from an ammoniacal solution with sodium carbonate, and instantly decanting from the first-formed precipitate. The Zn salt comes down a few seconds later.

Calcium Group. Precipitation is accomplished from a weakly ammoniacal solution. The drop must be dilute if good crystals are to be obtained, and warming slightly is often advantageous. The reaction is of value if one member only of the group is present, and hence is of most use as a confirmatory test. Calcium gives small rhombs, but with a large excess of reagent a double carbonate is formed, taking the form of thick prisms, if Sr and Ba are absent. Sr gives large globular-shaped masses, and Ba forms fine hairlike clusters, or small groups of crystals.

Sodium nitroprusside reaction. Crystalline precipitates are formed with Zn, Mn, Cd, Cu, Ni, Co, Fe, Hg^1. Mercuric salts, Ca, Ba, Sr, Sn, Sb, Bi, Al, Mg, Pb, and Ag give no crystals or precipitate even on evaporation. The solution must be neutral or slightly acid, and the test drop should be fairly concentrated.

ZINC. Isotropic spheres, irregular masses, or discs, which grow crystal faces on standing. If much mineral acid be present, small squares and thick rods may be formed.

CADMIUM gives rise to rough spheres and octahedra which polarize strongly.

MANGANOUS SALTS form a precipitate which is exactly similar to that of zinc.

COPPER gives an amorphous precipitate pale blue in colour.

NICKEL, amorphous pale green precipitate.

COBALT, amorphous pink precipitate.

IRON, on heating, forms a yellow sand.

MERCUROUS, amorphous mass.

Precipitations by metallic zinc. The metal in the form of a very small fragment is introduced into the drop. The solutions must be at the most faintly acid, and sodium acetate is best added as a precaution. A moderate concentration is required, and the solutions must be cold. The metals higher in the electro-chemical series are separated, and often present a sufficiently distinct appearance to enable the analyst to identify them.

(+) Mg, Al, Mn, Zn, Cd, Fe, Tl, Co, Ni, Sn, Pb,
H, Cu, As, Bi, Sb, Hg, Ag, Pd, Pt, Au...(–)

TREES are produced by lead, silver, and tin. The lead tree has a long straight "trunk" with side "branches" protruding at right angles, and "leaves" of a fern-like shape. Silver is very similar to lead, but is very "silvery" and metallic, more delicate, and has branches at all angles. Tin resembles silver, except that the branches are parallel to each other, and their formation is slower.

The remaining metals give results which are best described as moss-like.

Bismuth forms black feathers with peculiar curving and sharp-pointed ends, growing very rapidly, and quite characteristic. Antimony gives a similar tree, but more stodgy, and less curved or feathery. Cobalt forms a definite deposit only with difficulty, and nickel still less readily. Copper gives a characteristic appearance of thick masses with blunt angled ends.

Group reagents for acid radicles.

THE SULPHUR ACIDS.

Sulphides. Sulphuric acid distillation : silver nitrate : lead acetate : sodium nitroprusside.

Thiosulphates. Copper sulphate.

Sulphites. Starch-potassium iodide.

Sulphates. Calcium acetate : silver nitrate.

THE CYANOGEN ACIDS.

Cyanides. Sulphuric acid distillation : sodium picrate : silver nitrate : sodium bicarbonate distillation.

Thiocyanates. Ferric chloride : double thiocyanate (Zn, Hg).

Cyanates. Sulphuric acid distillation : cobalt acetate.

Ferricyanide. Sulphuric acid distillation : benzidine hydrochloride-ferric chloride.

Ferrocyanide. Iron salts : copper salts : quinoline HCl.

THE HALOGEN ACIDS.

Chlorides. Lead nitrate : silver nitrate.

Bromides. Starch-persulphate : silver nitrate.

Iodides. Starch-persulphate : silver nitrate : lead nitrate.

Chlorates. Partial oxidation to perchlorate by repeated evaporation with concentrated sulphuric acid.

Perchlorates. Rubidium chloride.

Iodates. Starch-morphine sulphate.

OTHER ACIDS.

Acetates. Silver nitrate : mercurous nitrate : uranyl nitrate + sodium chloride.

Arsenates. Silver nitrate : zinc acetate : ammonium molybdate.

Arsenites. Silver nitrate.

Borates. Ammonium fluoride : turmuric-viscose.

Carbonates. CO_2 production : lead acetate.

Chromates. Silver nitrate : strontium acetate : manganous sulphate.

Bichromates. Silver nitrate: strontium acetate.
Oxalates. Strontium acetate: silver nitrate: lead nitrate.
Phosphates. Ammonium molybdate: magnesium acetate.
Silicates. Ammonium fluoride.
Tartrates. Calcium acetate: silver nitrate: potassium salts.

Silver nitrate.

(*a*) ACIDS WHICH GIVE NO PRECIPITATE

Chlorate, fluoride, and nitrate. The following acids may give precipitates from concentrated solutions: silicofluoride, perchlorate, sulphate.

(*b*) ACIDS WHICH GIVE A DISTINCTLY COLOURED PRECIPITATE

1. Yellow. *Arsenite.* The test drop is preferable faintly ammoniacal. The precipitate is at first amorphous, but later crystallizes in many forms, amongst which are very small needles, clusters, and thin rods with V-shaped ends. The precipitate is soluble in acids and in ammonia.

Phosphates. The precipitate should be taken as a guide only, and a confirmatory test applied.

2. Violet. *Manganates* and *permanganates.*

3. Red-brown. *Arsenates.* Ag_3AsO_4 is precipitated, at first as a powder, thin plates, more or less hexagonal, being deposited later. The colour varies from reddish yellow to black, according to the thickness of the crystals. It should be noted that the solution must on no account be alkaline. Crystals are not often obtained very well developed, but they are readily recrystallized from hot dilute nitric acid. The salt is insoluble in acetic acid. Recrystallization may also be performed by solution in ammonium hydroxide, but the crystals obtained are a different compound, having the formula $Ag_3AsO_44NH_3$.

Chromate and Bichromate. Both are precipitated as a deep red chromate, from weakly acid solution. The crystals have the composition $Ag_2Cr_2O_7$, and take the form of thin rectangular plates and scales. If the solution is not sufficiently acid, small, almost black needles may be obtained. The precipitate is best recrystallized from dilute nitric. It is also readily soluble in ammonia, but crystallizes from this as needles.

Ferrocyanides give a yellowish or brownish red precipitate which is not often crystalline.

4. Brown-black. *Sulphides.* Soluble sulphides give an almost black precipitate.

(*c*) ACIDS WHICH GIVE A WHITE OR CREAM PRECIPITATE

1. Soluble in Dilute Nitric Acid.

Borates. Granular or amorphous.
Carbonates. Amorphous.
Cyanates. Amorphous.
Iodates. Amorphous, or crystalline in small needles and stars.
Oxalates. Granular at first, soon crystallizing in a variety of forms, short thick prisms or hexagons predominating.
Sulphites. Prisms, or granules.
Tartrates. In dilute solutions, small square crystals and short thick prisms; in concentrated solutions, granular precipitate.
Thiosulphates. Amorphous, changing through yellow to dark brown owing to a silver sulphide being formed through decomposition. Some sulphur always separates.

2. Insoluble in Nitric Acid.

Ferrocyanide. Turns brown by the action of nitric acid.
Chloride. Amorphous. May be recrystallized from ammonium hydroxide. (See previous description under HCl.)
Bromide. Soluble in ammonium hydroxide.
Iodide. Amorphous, pale yellow, insoluble in ammonia.
Hypochlorites. Amorphous.
Nitrites. Tangled needles, faintly greenish by reflected light.
Iodates. Amorphous, pale yellow.
Sulphates. Very characteristic precipitate. (See under Sulphuric Acid above.) The test may be made more definite by adding a trace of potassium chromate and nitric acid first, when the silver sulphate will be stained yellow by solid solution of the silver chromate, and the crystals will be somewhat larger in size.

Barium chloride. Few reactions of importance as individual identity tests are given, but the reagent is of value as a group test.

(*a*) NO PRECIPITATE

Acetate, bromide, chlorate, chloride, cyanide, cyanate, ferrocyanide, iodide, nitrate, nitrite. From concentrated solution precipitates may be obtained with arsenate, borate, ferrocyanide, oxalate, and chloride.

(*b*) PRECIPITATE INSOLUBLE IN NITRIC ACID

Silicofluorides, sulphates. Slowly soluble: chromates, bichromates, iodates.

(*c*) PRECIPITATE SOLUBLE IN NITRIC ACID

1. Amorphous or Granular. Arsenites, fluorides, phosphates, tartrates.

2. Amorphous, later Crystalline. Carbonate, chromate, bichromate, sulphites.

3. Crystalline. Iodates (stars), cyanates, in fairly concentrated solution (prisms).

Starch reactions. By the addition of starch to a suitably prepared drop of solution, tests for various acids may be obtained, according to the process employed, owing to the starch absorbing free halogen to give a characteristic colour.

1. AMMONIUM PERSULPHATE TEST. A trace of dilute sulphuric acid is added to the test drop, the whole is warmed slightly, and care taken to cool it thoroughly. A few grains of starch are added, farina preferably, followed by a very small crystal or drop of freshly prepared solution of ammonium persulphate. The starch granules should be examined at once as the colour is somewhat fugitive.

The starch granules are turned yellow by bromides, blue by iodides, and not changed by iodates under these conditions.

The following precautions should be observed. If the preliminary heating is carried on too long, some of the volatile acids set free will be lost. If too much persulphate or sulphuric acid is present, the starch may be destroyed for the purposes of this test. The drop must be cool when the starch is added, or the colour if produced will be immediately destroyed.

2. MORPHINE SULPHATE. If this compound be used in place of ammonium persulphate, iodates will turn the starch blue, iodides giving only a trace of free iodine, and bromides no blue coloration.

3. POTASSIUM IODIDE. A little of this compound is added to the drop, followed by a few starch grains, and finally the drop is acidified with a little sulphuric acid. The starch grains will be turned blue if nitrites are present, as a result of the iodine set free by the oxidation of HI by nitrous acid. A blank test should be made with the reagents.

4. POTASSIUM IODATE. A drop of potassium iodate is slightly acidified with sulphuric acid, and a few starch grains added. No colour should be produced. If this is the case, a little of the substance to be tested is now added. Sulphites, and thiosulphates liberate iodine, which colours the starch blue.

Sulphuric acid reaction. The chief value of this reagent lies in its power to liberate certain acids as gases. The following acids are volatile with sulphuric acid: acetate, borate, bromide, chlorate, chloride, cyanate (giving CO_2 and $(NH_4)_2SO_4$) ferrocyanide and ferricyanide (giving HCN) iodide, and nitrate.

Where doubt exists about the gas being evolved or not, a little of the solution should be evaporated to dryness with as little heating as possible, and covered with a drop of 1 per cent gelatine. When this has set, a little sulphuric acid is added; it gradually soaks through and the gas liberated is imprisoned in the gelatine and very easily observed. Carbonates give carbon dioxide in the cold.

A little of the substance should be distilled with sulphuric acid, by one of the methods mentioned in the previous article, and the gas evolved treated with a little of the appropriate reagent.

SILVER NITRATE. A cloudiness, or under favourable conditions, small white prisms, denotes cyanides or ferrocyanides. A black precipitate is caused by H_2S.

LEAD ACETATE. A white crystalline precipitate is given with carbon dioxide from carbonates and oxalates; sulphides form a black precipitate.

SODIUM PICRATE. HCN forms a blood-red coloration.

SODIUM NITROPRUSSIDE. This well-known test is employed for sulphides, a purple coloration being produced.

It should be noted that the examination of the residue after distillation may show ammonia to have been formed, which denotes the presence of cyanates.

Lead acetate. CARBONATES. Crystals of small rhombic prisms, or needle clusters.

CHLORIDES. In slightly acid solution, long needles. In neutral solution basic chlorides may be formed.

IODIDES. In slightly acid solution, hexagonal plates.

OXALATES. Large prisms, after a short time.

Other reagents. The following reagents test for only one or two acids, and are resorted to as confirmatory tests after some indication has been obtained by means of the group reagents already given.

AMMONIUM MOLYBDATE. A test drop of the substance is made acid with nitric acid; and a drop of ammonium molybdate solution acid with nitric acid, is run in. The drop is warmed gently. A yellow precipitate is obtained with phosphates, arsenates, and soluble silicates. The phosphate precipitate is amorphous at first, but often crystallizes later in octahedra.

ZINC ACETATE. A test drop is made slightly ammoniacal, after a little ammonium chloride has been added. A drop of zinc acetate added to this solution will cause arsenates to be precipitated in a crystalline form isomorphous with ammonium magnesium phosphate.

Ammonium zinc phosphate is not precipitated in a crystalline condition.

STRONTIUM ACETATE. Chromates come down from neutral or slightly alkaline solution, but not from acid solution. Dumb-bell like masses are obtained from ammoniacal solution. Oxalates are also precipitated as small octahedra and square plates. The solution must not be hot.

MANGANESE SULPHATE. In very faintly acid solution, manganese chromate is precipitated.

MAGNESIUM SALTS (acetate). Give precipitates with arsenate and phosphate.

CALCIUM ACETATE. Sulphates from faintly acid solution give the characteristic prisms.

Tartrates from faintly-acid solution give large, well-developed crystals, which are often slow in formation.

COPPER SULPHATE. A moderately concentrated drop of the reagent is taken, and a drop of the substance run into it at one side, gently. Sulphites give only a faint cloudiness, thiosulphates give a brown precipitate of copper sulphide, together with yellow crystals of copper thiosulphate.

NITRON SULPHATE. (Diphenylendanilodihydrotriazol-sulphate) in dilute acetic acid solution gives a tangled mass of very small crystals immediately. Later, bundles of prisms are produced, slightly brownish by reflected light. Iodides and bichromates should be absent for this test; the precipitate with the two mentioned radicles is soluble in warm water, but the nitrate is not. The solution should be fairly dilute, as concentrated ones may cause bichromates, ferro- and ferri-cyanides, chromates, chlorates, phosphates, oxalates, and tartrates to be precipitated.

MERCUROUS NITRATE. From concentrated solutions of acetates, plates and thin hexagonal prisms are formed. Sulphates give needles.

URANYL NITRATE. A drop of uranyl nitrate is mixed with a drop of the unknown substance, and a crystal of salt (NaCl) is placed in it. The reaction is the reverse of the test for sodium and is a good test for acetates.

AMMONIUM FLUORIDE. As already mentioned, these tests must be made upon a slide coated with celluloid, and a small piece of cover glass must be attached to the objective by means of a little glycerine, to prevent the glass from being etched. The slide should be removed from the microscope as soon as possible.

A moderately concentrated drop of the unknown solution acidified with HCl is treated with the reagent, a small crystal of NaCl being finally added. Borates give a precipitate, but silicates and titanium must be absent.

Silicates form very pale rose six-sided plates or prisms.

RUBIDIUM CHLORIDE. With perchloric acid, colourless crystals are obtained, which become coloured pink by absorption of potassium permanganate. The potassium or ammonium salts may be used in place of rubidium, though they are much more soluble. The ammonium salt is less good than the potassium.

COBALT ACETATE. A small crystal added to a solution of a cyanate causes immediately a deep blue ring to be formed, surrounded by a blue precipitate. As the crystal dissolves, the ring of crystals increases in diameter, and finally the whole drop is deep blue without any precipitate, which is soluble in excess of the reagent. On standing, deep blue prisms are deposited.

Thiocyanates also give a blue solution, but any crystals which may be obtained are different in form.

Cyanides give a brown coloration.

BENZIDINE HYDROCHLORIDE. Ferrocyanides in sodium acetate solution form light blue prisms and stars. Sulphates form white glistening plates; the reaction has been used for the quantitative estimation of sulphate.

FERRIC CHLORIDE. Thiocyanates give a deep blood-red coloration; ferrocyanides, a deep blue precipitate; ferricyanides, no precipitate or coloration in dilute solution.

COPPER SULPHATE. Ferrocyanides in slightly acid (acetic acid) solution, give a brown amorphous precipitate. The double thiocyanate reaction may be used as a test for thiocyanates, by adding mercuric chloride and zinc sulphate to the suspected drop. Traces of copper, of course, make this test more sensitive.

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CHAPTER VIII

ORGANIC MICROANALYSIS

THE identification of an unknown organic substance, or of a mixture of two or more such bodies, is often a task of considerable difficulty even when sufficient material is available for the carrying out of all desired tests and reactions. This difficulty is greatly increased in proportion as the quantity of material diminishes, so much so that even by the employment of microchemical methods a definite recognition is far too often impossible.

This state of affairs arises, of course, from the multitude and diversity of the known organic compounds, and the absence of any clear-cut method of analysis as is available when dealing with inorganic material; together with the increased difficulty in the purification and handling of minute particles of substance, and the limitation on the number of tests which may be applied.

Within recent years the microchemical identification of organic compounds by the crystalline form of their derivatives has been given greater attention than in the past, but even to-day, the crystalline form is not usually exactly described in the literature, some such term as "glistening needles" being of little value. The length of time required to record the optical properties of a new organic compound is so small that it might well be made part of the normal routine of description, taking its place beside the melting point, etc.

Because of this, and the additional fact that the crystalline form is sometimes liable to variation according to the solvent from which the body is crystallized or precipitated, typical microchemical tests of the kind exemplified in the previous chapter, rather fail in their general application. The choice has then to be made, in organic microchemical analysis, between two distinct methods, the one being to follow out the normal procedure for the identification of this class of substance, but using the special methods as are described in Chapter VI; the other method being to examine the crystalline form of certain derivatives, many of which are described in the following pages. Whichever method be adopted, the possibilities should always be narrowed down as far as possible by physical tests (such as melting point), and certain general group tests (such as the nitrous acid test for amines), before the more specialized microchemical reactions are carried out.

Physical methods have the advantage that the substance is, as a rule, recovered unchanged, and may thus be used for other tests, a most important point when economy in material is of such importance. Solubility in various solvents gives much information of a general kind with the minimum loss of material (see Clarke's *Handbook of Organic Analysis*), whilst the number of substances which sublime is sufficiently small to make this property of value.

Furthermore, the physical characteristics of crystals, for example the refractive index, and the behaviour under polarized light, may become of value even when these properties cannot be found described for the compound with which it is suspected that the unknown is identical; when the search has been narrowed down to a few possible compounds, it is then practicable to ascertain such characteristics for these compounds for comparison with those of the unknown.

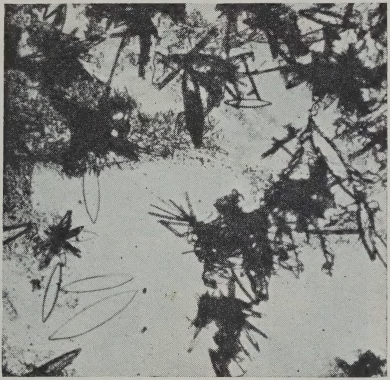

FIG. 111. ZINC SALT OR SCHAFFER'S ACID

Similarly, the microchemical reactions of such a list of compounds may quickly be determined, their choice being suggested by a perusal of the reactions given later in this chapter. An example of this method of approach is the crystalline form of some heavy metal salts of certain naphthalene sulphonic acid derivatives, illustrated in Figs. 111 and 112. (*J. Soc. Dyers and Col.*, 1927, p. 12.)

The method outlined below depends upon the exact determination of at least one physical constant, such as the melting point. If a further constant, such as the boiling point or the refractive index, can also be taken, these two in themselves are very often sufficient to point to one particular compound, which may then be tested for more closely. An example is paradichlorbenzene. The criticism may be made that the method is empyrical, but this is of little importance when a fragment of substance must be identified in any way possible. A more important objection, which will quickly be discovered in practice, is that the melting point of any substance not well known (and, indeed, of many which are frequently encountered), as given in the literature by various authors, may vary by a few degrees, and it is not always safe to take the highest recorded temperature.

It is also essential to have a sharp melting point, which presupposes that the substance is pure, or, in exceptional cases, is an eutectic mixture.

In the first place, then, the melting point is taken. The next step is to write down the substances melting around this temperature, going three or four degrees both above and below. The lists given in the *Chemiker Kalender*, or other year books, the International Critical Tables, and Mulliken, are invaluable, but they must be supplemented by each worker with the constants of compounds occurring more or less exclusively in his own work, such as plasticizers in the varnish industry, or intermediates in the case of dyestuffs chemistry. The resulting list will contain, perhaps, as many as 3,000 names.

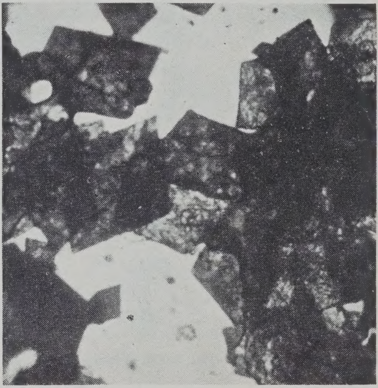

FIG. 112. ZINC SALT OF NAPHTHIONIC ACID

It is now necessary to eliminate, by all considerations available, a number of the substances which may possibly be present; the further tests made will depend entirely upon the circumstances, but it will usually be necessary to test for amino and hydroxy groups; if enough can be spared, a test for constituent elements is always worth the expenditure of material.

By these means, there should normally remain only two or three compounds, one of which is fairly probable. An attempt may then be made to prepare a derivative with a melting point; or one of the tests depending on crystalline form may be carried out.

When the substance available in small quantity is impure, giving no sharp melting point, and being in too small amount to purify successfully; or when any complexity of structure is encountered, which renders its inclusion in the more usually available melting point or boiling point lists improbable, the problem becomes almost impossible of solution. The most that may be done without the aid of good fortune is to gain some idea of the general character of the substance by preliminary tests, to eliminate the improbables, and finally to risk the remainder of the substance for a test on a somewhat larger scale for the particular substance regarded as being the most likely.

Separation of mixtures should be carried out as far as possible by means of solvents; though the separation is not so sharp as by chemical means, the substance is left unchanged by unsuccessful attempts.

The analysis may now proceed in one of two ways. Either a suitable derivative may be obtained in a state of purity, and its melting point determined as in ordinary organic analysis; or a microchemical test may be made, depending for its result on the crystalline form[1] of the precipitate observed. The former method is, in the present state of knowledge, almost essential in the case of the lesser known compounds, but for the more familiar organic compounds, the microchemical tests may be applied with confidence.[2]

Examination for constituent elements. NITROGEN. The ordinary organic ignition method is adapted for microchemical work, by using thermometer tubing with a bore of about 1 mm. as the ignition tube. A small fragment of sodium is pushed down to the bottom of the tube (which is, of course, sealed at the lower end) by means of a platinum wire or a thin thread or fibre of glass. The substance is then introduced into the tube by the same means as when taking a melting point, and finally a further small quantity of sodium is placed on the top of the substance. The tube is heated in a Bunsen flame in the ordinary way until the glass almost melts, and is then plunged red-hot into a micro-flask half-filled with water. The mixture is filtered by the pipette method and a small quantity of fresh sulphate solution added to the filtrate in a second micro-flask. The ferrous mixture containing the precipitate of ferrous hydroxide is heated to the boil and cooled, and a drop of ferric chloride is added. On the addition of excess of hydrochloric acid, a precipitate or blue coloration of ferric ferrocyanide is produced when nitrogen is present. The precipitate often only makes its appearance after some time. Preliminary evaporation is often required in place of the filtration in this test; the solution may be centrifuged if desired.

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[1] In the following pages reactions are grouped under the reagent heading. When the reactions of an individual substance are required reference should be made to the general index at the end of the book.

[2] By far the most complete account of the microchemical reactions of organic compounds is to be found in Behrens-Kley: *Organische Mikrochemische Analyse.*

A second, and perhaps more sensitive test, is to convert the nitrogen into ammonia. A capillary tube with rather thick walls is sealed at one end, and a little lime, slaked in moist air, is pushed down to the bottom. The substance is then added, and again some slaked lime. An asbestos plug is now pushed into the tube, to within a millimetre of the second grain of lime. A small conical plug of filter paper, the point only of which has been dipped in red litmus solution (containing an equivalent of $\frac{N}{10}$ acid) is pushed up the tube, point first, to within 2 cm. of the asbestos plug. The tube is now heated until the part containing the solid fuses up. The ammonia given off passes through the asbestos plug and turns the point of the filter paper blue. This test is very sensitive; the amount of substance to be employed is, as given by Emisch, two of the particles just visible to the eye. The lime should be tested by a blank test.

HALOGENS. A portion of the filtrate from the nitrogen base is boiled with excess of nitric acid for some time to remove the other nitrogen from the solution. After cooling, silver nitrate is added. Any precipitate indicates the presence of halogens. When only small quantities are present, the solution should be allowed to stand in the settling tube; the examination of the halide is carried out (for the determination of the halogen present) by ordinary inorganic methods. A further sensitive test is to introduce a little powdered copper oxide into a platinum wire loop. This is heated to make it sinter together, and a little of the substance is dropped on and heated in a Bunsen flame, first in the outer and then in the inner zone. A green flame indicates the presence of halogen, but certain other substances amongst which are oxy quinoline and urea are said also to give a green flame.

PYRIDINE TEST FOR SIDE-CHAIN HALOGENS. Carefully-filtered solutions must be used. The supposed halogen compound (the halogen not being in the nucleus) is boiled for 15 min. under microreflux with pyridine, cooled, water added, filtered, acidified with nitric acid, placed in the settling tube, and silver nitrate solution added. Standing for a few minutes concentrates any precipitate due to ionized chlorine in the hanging drop. The test is extremely sensitive, if the drop is touched off on to a slide, and examined under dark-ground illumination for cloudiness. The air must be free from HCl, and the settling tube is best supported in a wide-mouthed flask containing a little caustic soda.

SULPHUR. A portion of the filtrate from the nitrogen test is mixed with a boiled solution of sodium nitro prusside. Sulphides produce a violet coloration. A further test is to convert the sulphur into sulphate by fusing with up to ten times the quantity of a mixture of one part of potassium chlorate to five parts of sodium carbonate. When no action is visible, dissolve in water and allow to stand in a settling tube after adding HCl and $BaCl_2$.

Reactions with quinones. TETRACHLORQUINONE. This important reagent gives reactions of value with a great many amines. The addition compounds are made in all cases by dissolving the reagent and substance in benzene, or using concentrated aqueous solutions, and if no precipitate is produced, allowing the solution to evaporate somewhat. Polyphenols produce colorations or crystals by melting with the reagent or allowing the reaction to take place in a warm concentrated solution. The colour should be noted after the addition of water; acetic acid is then added, and the solution warmed. This usually results in the colour being destroyed and crystals precipitated.

Aniline: dull violet solution, quick ppn. of brown rectangular plates, and boat shapes, occasionally in pairs. o. anisidine: blue solution, colourless dendritic masses. p. anisidine: blue solution, yellow-brown hexagons. Catechol: (conc. soln.) dull blue, brown on dilution; HAc, on warming, gives colourless solution and crystals. Pseudo cumidine: blue solution, rhombic plates. Diphenylamine: no reaction of value. Dimethyl aniline: very characteristic dark blue solution, later long blue-black prisms, strongly dichroic. Eugenol: red-violet solution; HAc gives no pp. Guaiacol: red violet solution, HAc. gives no pp. Methyl aniline: no reaction of value. α naphthylamine (free base in nitro benzene soln.): green solution, acute-angled lattices of long green-yellow dichroic needles. β naphthylamine: no reaction. p. phenetidine: blue solution, irregular crystals. Pyrogallol: as catechol. Resorcinol: no change. o. toluidine: blue-grey solution, yellow-brown plates and prisms. m. toluidine: violet solution, yellow spear-head crystals. p. toluidine: blue solution, yellow-brown hexagons. m. xylidine: blue-green solution, later yellow. Boat-shaped crystals, later plates (72°). β tetrahydroquinoline: green solution, no crystals. Thallin: green solution, no crystals.

ALLOXANTIN. Quinoxaline derivatives are formed with ortho diamines in the presence of sodium acetate. Thus, o. phenylene diamine is precipitated in yellow needles.

BENZOQUINONE. Addition products are formed by dissolving the reagent in benzene, acetic acid, or alcohol, together with the substance, the reagent being in slight excess; on evaporation, crystals are formed with numerous phenolic bodies, noteworthy for their strong dichroism.

Catechol: mostly decomposed; some characteristic large red rhombic plates of quinine. p. amino dimethyl aniline: violet solution, alcohol pps., small crystals. Hydroquinone: (reagent solid) black deposit on reagent, almost completely ppd. α naphthol: long prisms and plates on evaporation. β napththol: ill-formed orange-red plates. Pyrogallol: (+ HAc) small yellow rods, dichroic (black and brown). Resorcinol: (in benzene + alcohol), rectangular plates of quinhydrone, yellow-red.

α DINITROPHENANTHRENQUINONE. A group reagent for aromatic hydrocarbons. The reaction is carried out in a small drop of concentrated nitrobenzene solution, by warming on the slide. On cooling, the derivatives crystallize out; they are usually highly coloured, and often dichroic. Acenaphthene: orange spikes and rhombs. Anthracene: greenish-blue plates. Chrysene: red sheaves of fine needles. Carbazole: characteristic red-violet or black-violet plates (83°), not dichroic. Fluorene: red-brown needles, thick hexagonal prisms. Naphthalene: yellow rhombic plates. Pyrene: red-brown needles, thick hexagonal plates. Phenanthrene: as pyrene.

α NAPHTHOQUINONE. Addition products are formed with phenolic bodies, in benzene solution. The quinhydrones crystallize easily after slight evaporation. α naphthol: bright red prisms, dichroic. β naphthol: no reaction. Hydroquinone: (aqueous solutions or slightly acid with acetic acid) dichroic plates, on warming.

β NAPHTHOQUINONE. Reacts with ortho diamines. The hydrochloride of the base is employed, in presence of sodium acetate, the derivative being recrystallized from weak acetic acid, or nitrobenzene. Phenylhydrazine in dilute acetic acid solutions gives a red coloration; the test is sensitive. Carbazole precipitates red crystals of its derivative, mixed with the yellow crystals of the reagent.

PHENANTHRENQUINONE. Ortho diamines react in dilute acetic acid solution, the derivatives being crystallized from hot dilute acetic acid or nitrobenzene. o. phenylene diamine hydrochloride: forms small yellow spikes and needles. By warming with a concentrated solution. Carbazole (in nitro benzene): glittering orange-red rectangular plates or hexagons are formed. The reaction is sensitive and characteristic.

β DINITROANTHRAQUINONE. Anthracene: red-violet needles. Carbazole: blue-violet needles. Chrysene: red needles. Pyrene: red needles.

Oxidation. POTASSIUM PERMANGANATE. The activity decreases in the order acid, neutral, and alkaline solutions. A mildly-alkaline solution or suspension of the substance is brought to the boil in an anilide tube, and permanganate is added a drop or two at once until the oxidation is complete. The oxidized mixture is filtered from the precipitated manganese oxide by means of a filter pipette (plugged with asbestos) or glass wool, the filtrate concentrated, and extracted with ether from either acid or alkaline solution. Some oxidation products remain admixed with the oxide. Typical examples of reactions are: Benzophenone: no change on boiling. Acetophenone forms potassium benzoate and potassium glyoxylate. The acid oxidation is as a rule too drastic for analytical purposes.

ACID AMMONIUM BICHROMATE. This provides a very useful general oxidizing agent. It is used in aqueous solution, employing heat if required.

POTASSIUM BICHROMATE. Benzidene: cloudy, later dark blue pp., recrystallized hot water, felted mass of small needles. Thallin: (acid) green coloration; (excess reagent) orange solution, ill-formed sheaves and rosettes of hairs and curved needles.

POTASSIUM FERRICYANIDE. A further and most useful special oxidizing agent is potassium ferricyanide. Its use depends upon the fact that its quinoline salt is rather soluble, crystallizing in yellow spikes, whilst the quinoline salt of the ferrocyanide is insoluble. Therefore, by warming a mixture of quinoline, potassium ferricyanide, and a small excess of HCl, with, e.g. an aldehyde, colourless cubic crystals of the quinoline ferrocyanide are produced on cooling, or possibly after allowing to evaporate slightly. This is a most sensitive test for the presence of reducing compounds.

FEHLING'S SOLUTION. The reagent provides a very sensitive test for reducing compounds when carried out in the following manner: A very small quantity of the substance is heated in a melting-point tube, in boiling water, for some time with a drop or two of Fehling's solution. After cooling, the tube is mounted in dilute glycerine. This is carried out by building up a long rectangular cell on the slide by means of thin rolls of plasticine, in which the glycerine is placed. The immersion of the melting-point tube in the glycerine enables the contents to be seen without difficulty, even under a moderately high power. Cuprous oxide is black by transmitted light, but by reflected light shows up as reddish (or orange) granules.

SILVER MIRROR. A slide is carefully polished, after cleaning with alcohol. A drop of ammoniacal silver nitrate is mixed with a small drop of the substance placed on the slide, and warmed without boiling. A mirror is not always formed, but a reducing substance will always produce a black precipitate.

$KClO_3 + HCl$ is a useful oxidizing mixture. o. cresol: chloroquinones. p. cresol: no chloroquinones.

CHROMIC ACID finds frequent application. Thymol: (+ HAc) thymoquinone (extract with benzene). Hydroquinone: (+ H_2SO_4 dil. cold) quinhydrone m.p. 171° C. Anisaldehyde: (+ HAc) evaporate to dryness and sublime. Benzaldehyde: (+ HAc) very easily oxidized to benzoic acid. Cuminol: brown mass. Cinnamic aldehyde: (+ H_2SO_4) careful oxidization gives cinnamic acid (sublimes), and HAc (test with uranyl acetate)

Reduction. For the present purpose *sodium hydrosulphite* is the most suitable reagent, for it gives a vigorous reduction, with absence of side reactions, whilst its own oxidation products are simple and not troublesome, apart from occasionally a little sulphur.

Hydrosulphite powder is added to a boiling solution or suspension of the substance in water or alcohol, under micro-reflux. The powder is added gradually, until the reaction is judged to be complete, and the mixture allowed to cool. The products may be so varied that it is almost impossible to give a detailed scheme for separation and identification. The melting point will usually have given some idea of the substances to be expected. In general, the cooled mixture should be filtered, giving amines insoluble in water, together with some amino-sulphonic or carboxylic acids of low solubility. The solution should then be evaporated down somewhat, salt added to saturation, and after settling for a little while, again filtered, giving amino acids and the like, of good solubility, but which can be salted out.

By now adding strong NaOH, bases which have a soluble hydrochloride will be obtained, whilst extraction with ether will give the more soluble amines such as diamines. By treatment with CO_2 and steam distilling or extracting with ether, amino phenols may be isolated. This general scheme requires, of course, more material than is often available when micro methods are called into use, but often the reduction on a larger scale produces an amount of substance which may only be identified by the methods outlined in these pages.

Hydrolysis. The reaction is best carried out with up to $\frac{1}{2}$ gm. of substance, boiling under reflux for as long as the hydrolysis required.

The usual reagents are *aqueous or alcoholic potash*, the latter giving a lower reaction temperature, but acting as a solvent for many compounds; and *concentrated HCl*. In general, the compound will be split up into one body soluble in ether; in absence of any result, steam distil. In the case of acid hydrolysis it will often be preferable first to make the mixture alkaline before extraction. The solution is then acidified, and filtered, extracted with ether, or steam distilled, according to circumstances. The solution should finally be brought to exact neutrality for the separation of amphoteric bodies.

It may be noted that the component which separates as a solid should be specially examined, as it is not difficult to purify sufficient for a micro-melting point determination to be made.

Acetyl compounds may be hydrolyzed with phosphoric acid, the reaction mixture being then steam distilled. The distillate is tested for acetic acid by means of the sodium uranyl acetate double salt. Hydrolysis by means of alkalis is also possible, the mixture after hydrolysis being acidified with phosphoric acid, and steam distilled, the distillate being tested as before. Chlorides do not interfere. Alkaline hydrolysis very often enables the amine to be steam distilled off simultaneously with the hydrolysis. The test may be simplified in many instances by merely evaporating to dryness with caustic soda, and testing the solution of the residue with uranyl acetate.

Long boiling with acids hydrolyses cyanides; the liquid should be made alkaline with NaOH and the ammonia distilled off. The reaction has not too wide an application.

Halogen derivatives. BROMINATION. Bromine water, or bromine in concentrated HCl, may be employed. The reagent is mixed with a solution of the substance in a suitable solvent. Derivatives often come down at first in the form of an oil, which is slow to crystallize. It is advisable to treat bromo derivatives with a little formic acid, in order to remove any slight excess of bromine. The reaction is often of value in distinguishing between isomers. Acetophenone: (+ HCl in alcohol), white needles and stars. Aniline: small needles, bigger from alcohol. m. amido benzoic acid: (in HCl), fine pp. of small stars. Catechol: destroyed. o. cresol: oily drops. p. cresol: oily drops. m. cresol: oily drops, later branched radial needles. p. amino dimethyl aniline: (Br in concentrated HCl), dibromo deriv. Guaiacol: red solution. Hydroquinone: quinhydrone, then quinone. Phloroglucinol: crystals of no value. Pyrogallol: reaction of no value. Pyridine: small fine needles. Phenol: fine needles, sheaves on surface, easily sublimes (needles). Resorcinol: rods. o. toluidine: oily drops. m. toluidine: small rods. p. toluidine:

oily drops. Thymol: oily drops. m. xylidine: oily drops. p. xylidine: oily drops.

Acid radicles. POTASSIUM FERROCYANIDE; in aqueous or dilute alcoholic solution. Acridin: (acid), yellow crosses and spikes. Antipyrin: moderately soluble yellow plates. Benzidine: small plates and stars. β collidine: thin rhombic plates (80°). Pseudo cumidine: pale red rhombic plates. Dimethylaniline: (weak acid), pale yellow pp., later quadratic plates (85°). Lepidine: less soluble crystals like quinoline. m. phenylene diamine: pale red rhombic plates. Pyridine: rather soluble yellow rhombic plates (75°) or prisms, extinction angle 33°. Quinoline: hot, slightly acid), yellow plates (70°) extinction angle 35°. Isoquinoline: slowly, monoclinic prisms, hexagons. Thallin: soluble, short yellow prisms. β tetrahydro quinoline: deep yellow cubes, hexagons. o. toluidine: slowly, long rods in stars and lattices.

IODO- AND CHLORO-PLATINATES. Chloroplatinic acid is on the whole less useful than iodoplatinic acid, which is prepared as required by adding sodium iodide to the reagent. A drop of the reagent is mixed on the slide with a drop of an aqueous or weakly alcoholic solution of the substance under examination. The more soluble double salts take a few minutes to form: this is quite a usual occurrence in precipitation methods, and it should not be concluded too hastily that no precipitate will form. The reagent has a wide application, and is one of the most useful available.

IODOPLATINATE. Acridine: on warming, reddish drops of no value. Aniline: small single black rectangular prisms, needles, stars. Betain: rectangular plates, right angled crosses. β collidine: slowly, short thick prisms. Diphenylamine: slowly, short thick rectangular prisms. Dimethylaniline: small thick acute-angled prisms. Methylaniline: acute-angled lattices of thin dentilated needles. α naphthylamine: thick plates. β naphthylamine: large rhombic plates, later spear-head shaped plates. Pyridine: characteristic insoluble rods and spikes. Picoline: long monoclinic curved spikes and plates. Quinoline: very small insoluble crystals. Isoquinoline: well-formed, acute-angled plates. β tetrahydroquinoline: small black crystals. o. toluidine: slowly, thick short rectangular prisms. p. toluidine: large sheaves of curved branched fronds, with thick ends. m. xylidine: ill-formed sheaves and rectangular prisms.

CHLOROPLATINATE. Benzidine: very characteristic lattices of insoluble needles, at right angles. Piperazine: soluble pale yellow plates (82°). Thiourea: (excess substance), squares and rectangles; (excess reagent), black balls and stars. Tyrosine: soluble derivative. Urea: no pp. cold.

NITRATION. Nitric acid of S.G. 1·5, or a mixture of one drop of concentrated sulphuric acid with four drops of concentrated nitric acid, may be employed. The reaction is carried out in an anilide tube. It is usually successful to heat for 10 min. in a water bath, after which a drop of fuming nitric acid may be added, and the mixture allowed to cool. The derivative is most easily obtained by running the mixture into water in a settling tube. It should be remembered that oxidation often takes place.

NITROUS ACID. A solution of the substance is obtained by warming with a little dilute HCl; if the amine does not dissolve, concentrated HCl should be used, and the amine precipitated from this solution by means of water in a very finely-divided condition. A crystal of sodium acetate is added, and a small drop of sodium nitrite solution run in under the usual conditions.

Observation under a 32 mm. objective will usually show traces of bubbles of nitrous acid. The drop should be allowed to stand for a few minutes, for in some cases (p. amines) the only sign of reaction is the solution of a previously insoluble body. A large drop of slightly alkaline or perfectly neutral (filtered) solution of β naphthol or R salt is now placed near the reaction drop; the latter is run slowly into the reagent drop, when a red or orange coloration probably indicates a primary amine.

Any other precipitate should be separated by filtering off the drop of liquid, and dried by means of filter paper. It is then separated into two halves. One-half is dissolved in a drop of concentrated sulphuric acid, a fragment of phenol added, and after solution, a drop or two of water. A little of the liquid is transferred to a clean slide, and a drop of concentrated NaOH added until the solution is alkaline. Any blue coloration due to nitrosamines may be seen very distinctly by means of vertical illumination, a piece of white cardboard being placed under the slide.

The other half of the precipitate is dissolved by gently warming in a drop of dilute HCl; a little is transferred to a cover glass, which is then inverted over a cell slide containing FeS and HCl. Reduction of a p. nitroso compound to a diamine takes place slowly; the presence of the p. diamine may be proved by the addition of a trace of ferric chloride. Nitrosamines on reduction regenerate the original amine; the reduction test described may be carried out by treating the nitrosamine with alcoholic HCl in an anilide tube, in order to produce a p. nitroso derivative (if the position is free).

The Sandmeyer reaction is important, especially for the diamines; the diazo body is in this case formed in a micro reflux, a little cuprous chloride dissolved in concentrated HCl is added, and the mixture is heated in a water bath. The resulting solution is steam distilled, which usually separates the chloro derivative.

Aliphatic p. amines evolve nitrogen when treated with nitrous acids, and after the reaction may be distilled from an alkaline solution, sufficient of the alcohol produced being carried over, as a rule, for the formation of some derivative of the phenyl urethane type. The amines themselves form good chromates and picrates, or double salts with mercuric chloride, which may be examined for their crystalline form.

Some typical reactions with nitrous acid follow.

NITROUS ACID. Antipyrin: green solution, blue-green plates (63°), strong double refraction. Catechol: faint brown pp. Carbazol: solid nitroso derivative m.p. 66, giving a p. nitroso body with special precautions. Dimethylaniline: para nitroso body, m.p. 85° C. re-crystallizes from 10 per cent HCl in pale green dichroic needles. Guaiacol: brown solution, no pp. Hydroquinone: oxidized to quinhydrone, gas evolved, yellow-brown solution. Methylaniline: oil, extract with ether, evaporate to dryness, add phenol + concentrated sulphuric acid, add dilute NaOH till alkaline, when a blue solution is obtained. α naphthol: yellow-brown powder. m. phenylene diamine: bismark brown, salted out by NaCl as a black pp. Pyrogallol: no reaction of value. Resorcinol: red-brown solution, later pp.

PICRATES. An alcoholic solution is allowed to react in an anilide tube, with or without heating. The point is broken, the liquid run into water, and the precipitate, after washing with a little water, is crystallized from aqueous alcohol. When the crystalline form only is to be observed, the reaction is carried out on the slide by mixing alcoholic solutions of substance and reagent.

Anthracene: red needles. Acenaphthene: orange needles. Carbazol: red needles. Chrysene: red needles. Fluorene: orange needles. Naphthalene: yellow needles. β naphthol: orange needles easily decomposed by sodium carbonate. α naphthol: dichroic needles, easily decomposed by sodium carbonate. α naphthylamine: fine sheaves of hair crystals. Phenanthrene: orange needles. Pyrene: red needles.

ACETYLATION. A good general process is to dissolve the substance in a mixture of acetic anhydride and glacial acetic acid, in an anilide tube. Heat in a glycerine bath, cool, break the point, and run the contents into a small watch glass. Neutralize with sodium carbonate, settle, filter off excess water with a micro pipette, wash the solid repeatedly with large drops of water, and re-crystallize from aqueous alcohol.

ANILIDES. A fragment of substance is heated with two drops of aniline in a glycerine bath. When the reaction is completed the capillary tube is broken, and the liquid run into a small watch glass, where it is washed well with dilute acid, and crystallized from aqueous alcohol.

SCHOTTEN BAUMANN DERIVATIVES. A little of the substance is placed in an anilide tube, and mixed with three or four drops of benzoyl chloride. About 1 c.c. of cold dilute NaOH is added, and the mixture is alternately stirred with a platinum wire and allowed to stand, until the smell of benzoyl chloride is gone. The liquid is allowed to settle, the top liquor removed by micro pipette, the capillary tube broken, and the solid blown into a small watch glass, where the water is allowed to evaporate. The solid remaining is recrystallized from alcohol in a second watch glass, and the crystals and liquid transferred into a settling tube. The solid settles into the drop at the bottom of the tube, and is touched off on to a slide. The alcohol is blotted off, and a further drop of alcohol added and removed. The melting point may now be taken.

Benzene sulphonyl chloride is a useful substitute for benzoyl chloride, for p. amine derivatives are soluble in the caustic soda, but may be extracted by means of ether. This is carried out by transferring the top liquor removed by the micro pipette, to a. settling tube, and adding ether. A mixture of s. and t. amines may be separated by dissolving the mass in benzene and extracting the t. amine with dil. HCl. Diphenylamine is not attacked by benzene sulphonyl chloride.

CHLORO- AND BROMOAURATES. These double salts are of value in the analysis of the pyridine, quinoline, and alkaloid groups, but have little application elsewhere.

CHLOROAURATE. Betain: yellow cloud, later yellow rectangular plates.

BROMOAURATE. β collidene: thin needles. Lepidene: thin needles. Pyridine: wide red-brown plates, prisms, strongly dichroic. α picoline: dendritic crosses, slightly dichroic. Isoquinoline: needles and spears.

POTASSIUM BISMUTH IODIDE. Aniline: soluble broad yellow prisms. Pyridine: (hot acid solution), very characteristic insoluble orange hexagonal plates, pointed needles. Quinoline: (hot dilute HCl solution), red granules, rods, needles.

SODIUM SULPHATE (in aqueous solution). Benzidine: (neutral), fine white pp. recrystallized from hot water in triangles and hexagons, strongly polarizing. p. phenylene diamene: slowly, irregular plates and stars. p. phenetidine: rectangular plates, sharp extinction.

Sodium Oxalate (in aqueous solution). Para toluidine: short colourless prisms, stars, small extinction angle, strong double refraction.

Potassium Tartrate (in aqueous solution). α naphthylamine: short prisms. β naphthylamine: long fat needles.

Ferric Chloride. The reagent may be employed in aqueous solution (or better, in solution in alcohol). It is chiefly of value in microchemical work by reason of the colour reactions which it gives with a large number of oxidizable compounds. In some instances the colour is produced only on warming; very often the colour is only transient; and in a few cases the colour is changed by excess of the reagent. p. anisidine: (acid), violet solution. Benzaldehyde: no coloration. o. cresol: violet solution. p. cresol: blue solution. Catechol: green flocks. Dimethyl p. phenylene diamine: violet solution. Eugenol: blue solution. Gallic acid: dark blue pp. soluble in excess to green. Guaiacol: green solution. Hydroquinone: transient blue solution. α naphthol: white pp. β naphthol: white cloud. α naphthylamine: (acid), blue flocculent pp. β naphthylamine: (acid), green grey flocculent pp. Orcinol: blue-violet solution. Phenol: violet solution. Phloroglucinol: intense transient violet solution. Protocatechuic acid: green; sodium acetate: changes to violet; sodium carbonate: changes to blue-red. o. phenylene diamine: (acid), red crystals of pp. diaminophenazine HCl. Resorcinol: blue-violet solution. Salicylaldehyde: violet solution. Salicylic acid: no pp. Thymol: transient green solution. Vanillin: pale blue-green solution; on warming, characteristic pp. of colourless needles of dehydro divanillin.

Calcium Salts: Lime water (or calcium nitrate). Acetic acid: very soluble salt. Butyric acid: woolly rosettes. Benzoic acid: soluble salt. Caproic acid: easily crystallized salt. Caprylic acid: rectangular plates. Catechol: stars and plates. Meta cresol: oily drops. Eugenol: felted mass of needles. Formic acid: plates, prisms (70–73°) insoluble in alcohol, soluble in water, parallel extinction, optically negative. Glyoxylic acid: rectangular prisms, slight extinction angle. Glycollic acid: soluble salt, later giving needles and hairs. Guaiacol: stars and rods. Malonic: moderately soluble salt. Oenanthylic acid: easily formed rectangular plates and long thin ribbons, not doubly refractive. Oxalic acid: long hexagons. Phenol: very soluble salt. Pyrogallol: violet solution. Phloroglucinol: cloudy solution. Proprionic acid: very soluble salt. Tartaric acid: rhombic plates ($57\frac{1}{2}$°). Isovalerianic acid: soluble salt.

Lead Salts. Lead acetate is usually employed. Acetic acid: salt is difficult to crystallize; thick monoclinic prisms insoluble in absolute alcohol. Benzoic acid: white pp., recrystallizes from lead acetate solution as small rods. Campheric acid: short thick prisms. Cinnamic acid: fine powder. Formic acid: rhombic needles, and rectangular prisms, optically negative, slight extinction angle, moderately soluble in water, slightly soluble in alcohol. Glyoxylic acid: pp. insoluble in hot water, soluble in ammonium acetate. Gallic acid: from very dilute solutions, gives yellow sphaeroids. Glycollic acid: no change. Hippuric acid: sheaves of thin needles. Malonic acid: white pp. recrystallizing from hot water in thin prisms, weakly polarizing, optically negative, slight extinction angle. Oxalic acid: right-angled crosses, and needles, black by transmitted light. Propionic acid: very soluble salt. Succinic acid: white pp., later small rhombic plates (70°–75°) optically positive. Salicylic acid: (boiled) small prisms (70°) extinction angle 42° to the longest side.

Silver Salts. Silver nitrate is employed in aqueous solution. Acetic acid: colourless rhombic plates and stars. Anthranilic acid: (the free acid is employed, a rod dipped in ammonia being held above the drop), stars of sycamore leaf shaped groupings. m. amido benzoic acid: slowly, acute-angled prisms in stars. Butyric acid: small sparse feathers and needles. Caffein: small prisms. Cinnamic acid: fine powder. Citric acid: square and boat-shaped crystals. Caproic acid: sphaeroids. Formic acid: fairly soluble irregular rosettes, darker in neutral or alkaline solution, gradually developing granules of silver in the crystals. Prisms show extinction angle of 70°. Glycollic acid: plates, prisms (60°) slight extinction angle; and prisms (40°) with 34° extinction angle. Glyoxallic acid: no crystals; reduced on warming. Glycocoll: no alteration until ammonia is added cautiously when crystals with blue colour by reflected light, brown by transmitted light, are obtained (soluble in excess); weakly polarizing, slight extinction angle. Gallic acid: slow ppn. of silver. Hippuric acid: (ammonium salt) characteristic features. Isovalerianic acid: badly formed plate clusters. Malic acid: cloudy, later sphaeroids. Oxalic acid: rather soluble rectangular plates. Propionic acid: acute rhombic plates (72°) extinction angle 8°, and dendritic figures. o. phthallic acid: no precipitate, but sodium acetate added gives crystals. Phthalimide: brown needles. Succinnic acid: peculiar crosses, with spearhead-shaped limbs, two of which are longer than the other pair. Tartaric acid: thick square plates. Tyrosine: very small crystals. Theobromine: square and rectangular plates, strong negative polarization, extinction angle 20°.

Cerium Salts. Formic acid: very characteristic pentagonal dodecahedra, slowly forming from dilute solutions, negative polarization.

BARIUM SALTS. Acetic acid: very soluble. Butyric acid: woolly rosettes. Formic acid: crystals insoluble in alcohol. Propionic acid: (from a concentrated solution of the Ca salt) octahedra, double pyramids (62°). The reaction can also be carried out by dissolving solid calcium propionate in a drop of barium acetate.

COPPER SALTS. Alanin: stars and thick rods are ppd. on addition of alcohol. Asparagin: very characteristic. Add alcohol in small quantities, warming for 20 sec. after each addition, until very small boat-shaped prisms and crosses with thick branched arms are formed. Ortho amidobenzoic acid: rhombic pseudohexagonal plates, strongly polarizing. Meta amidobenzoic acid: mossy green dendritic figures. Para amidobenzoic acid: moderately soluble prisms. Butyric acid: (moderately strong solution of Ca salt) green flocks. Dilute solutions after evaporation give a green oil which later forms long hexagonal prisms (90°) slightly dichroic, extinction angle 40°. Citric acid: rather soluble stars. Glycollic acid: insoluble short acute-angled prisms (73°) not dichroic, negatively polarizing, extinction angle 26° with prism diagonal. Glycocoll: stars of thick rods, rectangular prisms, blue reflected light, brown transmitted light, weak negative polarization, slight extinction angle. (It is necessary to add ammonia after the copper sulphate.) Isovalerianic acid: green drops; from 10 per cent alcohol, dark green monoclinic hexagonal and rectangular plates and prisms are obtained, not dichroic; the lower acids do not interfere. Phthalimide: sheaves of black needles. Picolinic acid: long light blue rhombic plates, characteristically dichroic, ext. angle 43°. Tartaric acid: fine powder. Tyrosine: very small crystals.

ZINC SALTS. Camphoric acid: small rectangular prisms. Lactic acid: small colourless needles and prisms, refractive index so near to that of water that the crystals can only be seen by polarized light. Ortho phthallic acid: hexagonal plates.

BISMUTH SALTS. Citric acid: crosses and boat-shaped prisms.

THALLIUM SALTS. α naphthoic acid: curved needles. β naphthoic acid: rectangular crosses and rosettes. Orthophthallic acid: hexagonal plates, rectangular plates, double refraction, slight extinction. Para phthallic acid: (ammonia present) thin, very characteristic elliptical formations. Thiourea: long colourless prisms.

COBALT SALTS. Lactic acid: sheaves of thick reddish needles and thin prisms.

MERCURIC SALTS. (Mercuric chloride in aqeuous solution. Acetic acid: colourless stars, sheaves of thin needles, extinction angle 0°. Acridine: (moderately concentrated solution) long yellow rather soluble needles. Butyric acid: small sheaves of needles. Caffein:

long colourless spikes and prisms, extinction angle 32°. Beta collidene: very soluble. Isovalerianic acid: small well-formed rectangular plates. Lepidene: like quinoline, but less soluble. Propionic acid: thin feathers and sheaves of needles. Pyridene: large soluble colourless needles; later prisms (69°) strong negative double refraction, extinction angle 10°. Alpha picoline: more soluble needles of the pyridine type, single. Quinoline: (acid) single colourless needles, extinction angle 8°. Isoquinoline: long needles. Tyrosine: no pp. Thiourea: colourless needles and stars. Beta tetrahydroquinoline: (+ NaI) white needles, stars.

SOLUBILITY IN ALKALI. The method on page 91 is employed. The solution obtained is filtered, and one-half deposited on a clean cover glass, the other half on a clean slide. The cover glass is subjected to the action of HCl gas, (see page 107); the slide is treated with carbon dioxide, by placing it over a small watch glass containing marble and sulphuric acid, the whole being covered by half a Petri dish. The drops are examined for solid insoluble in neutral salt solution or in carbonate of soda.

Acid should not be added to the test drop directly, because many water soluble substances of the amine type are often found to give a precipitate, whilst many amphoteric substances are only soluble in water through a limited pH range.

Condensations and syntheses. PHENYLHYDRAZINE. Many valuable reactions are given with aldehydes and quinones. The reactions with sugar are well known and characteristic. Three methods are given below for its employment. The settling tube is a convenient method of obtaining crystals, or the anilide tube may be employed throughout the operation.

1. A 10 per cent solution of phenyl hydrazine in glycerine is placed in an anilide tube, with 0·5 grm. of sodium acetate, and 5 c.c. water; 0·05 grm. or less of the substance is then stirred in with a platinum wire. The tube is heated for half an hour or more, cooled by settling for some time, the precipitate run off through the broken capillary tube and recrystallized in a settling tube from hot water or alcohol.

2. The substance is dissolved in water or alcohol, and an excess of the following mixture added: 1 grm. phenylhydrazine, 1·5 grm. sodium acetate, 10 c.c. water. Heating on the water bath is usually required.

3. A very quick method. One drop of phenyl hydrazine, and two drops of acetic acid are added to a few milligrams of substance. After heating to the boil, water is added drop by drop until a precipitate is just perceptible, when a drop is taken out, placed on the

slide, and allowed to crystallize. Sugars do not give very good results by this method.

Anisaldehyde: slowly, rectangular plates and hexagons. Acrolein: pale yellow drops. Acenaphthenequinone: needles. Alloxantin: ill-formed yellow crystals. Alloxan: reduces to alloxantin. Acetophenone: oil. Acetone: oil. Benzaldehyde: colourless needles. Benzoquinone: oxidized. Benzil: yellow needles. Benzophenone: yellow oil. Cuminol: oil, later rhomboidal plates. Cinnamic aldehyde: oil, later small needles. Chloral hydrate: slowly characteristic yellow-brown needles. Furfural: (+ NaAc) drops, later small needles. Glyoxal: (HCl) warmed, flocculent yellow pp. later characteristic orange stars of the osazone. Glyoxalic acid: drops, later yellow rectangular prisms and crosses. Para hydroxy benzaldehyde: rather soluble rods. Isatin: (HCl) yellow insoluble needles. Laevulinic acid: (HAc) white pp., later hexagonal plates. β naphthoquinone: (HAc) red solution, later red needles. Phenanthraquinone: (HCl) red solution, later plates. Piperonal: yellow drops. Salicyclic aldehyde: very small plates. Vanillin: oil.

Diazotized Sulphanilic Acid, added to an alkaline solution of a phenolic body usually produces a yellow, orange, or red coloration. The colouring matter may be precipitated in a crystalline condition by the addition of NaCl or ammonium carbonate; HCl often produces a precipitate of value.

Catechol: deep yellow solution, NaCl: slowly, quadratic plates. Orthocresol: orange red solution, NaCl: thick dark yellow needles. Meta cresol: brown orange solution, NaCl: very small needles. p. cresol: deep red solution, NaCl: pale yellow stars and needles. Hydroquinone: no coloration, gas evolved. α naphthol: bright red solution, NaCl: needles and stars. Pyrogallol: brown solution. Resorcinol: yellow solution, NaCl: brown flocks. Thymol: yellow-brown solution, NaCl: complete ppn. in flocks.

Para Nitroso Dimethyl Aniline (employed in presence of sodium acetate). Catechol: yellow-brown rods. o. cresol: branched needles, rosettes (dilute solutions, rectangular prisms and plates.) Meta cresol: wide, yellowish-green, rectangular plates. p. cresol: pale brown needles and stars. Phenol: (saturated solution + NaAc) yellow plates. Thymol: (+ NaAc) fine needles. Resorcinol: stars and acute angled prisms. (Evaporated with excess HAc gives a dark blue pp. of indophenol.)

Urea (Carbamide) in aqueous solution with: Acetaldehyde: fine needles. Formaldehyde: (+ HCl) concentrated solutions give colourless rhombic plates and rosettes. Glyoxal: crystals, not characteristic. Semi-carbazide with: Anisaldehyde: like benzaldehyde.

Acetaldehyde: very soluble rhombic crystals. Acetophenone: (HCl) colourless needles and stars. Alloxan: (HCl + NaAc) colourless rectangular prisms. Acetone: no change. Benzaldehyde: colourless rods. Benzil: rosettes. Benzophenone: colourless squares. Cuminol: colourless masses. Furfurol: colourless rectangular plates. Formaldehyde: slowly, mass of fine needles. Glyoxal: not characteristic. Glyoxalic acid: oil. Laevulinic acid: (HCl) slowly, acute-angled rods. β naphthoquinone: (HCl) orange rosettes. Phenanthrenequinone: yellow needles and stars. Piperonal: colourless stars. Vanillin: crosses, needles.

HYDROXYLAMINE DERIVATIVES. 2–3 c.c. of alcohol are used to dissolve 0·05 gm. of the substance and 0·1 gm. anhydrous sodium acetate, in a large anilide tube which is fitted with micro reflux. 0·1 gm. of solid hydroxylamine is added, and the mixture heated for half an hour or so until the oxime is formed, which often separates as a solid. The liquid is run through the broken capillary tube into a deep watch glass, evaporated to dryness, and extracted with ether.

SODIUM BISULPHITE DERIVATIVES. An etherial solution of the compound is treated with an aqueous solution of sodium bisulphite in a settling tube. The carbonyl compound passes out of the ether to form the additive compound which usually separates out in the water layer. The compounds are of more importance for separation and purification than for identification.

PHENYL ISOCYANATE DERIVATIVES. Boil together equimolecular proportions of substance and reagent as rapidly as possible, but not in an open vessel, as this tends to the formation of diphenyl carbamide. Extract with benzol or ether to remove the unaltered reagent. Wash with cold water, and crystallize the derivative from alcohol, or a mixture of ether and petroleum ether.

TEST FOR CARBOHYDRATES. The tube shown in Fig. 93, which is due to Behrens, is employed for the detection of carbon dioxide produced by fermentation. The substance, together with a little yeast, is placed in the first bend, and the point of the tube sealed with a little vaseline. The second bend contains lime water.

The reaction may also be carried out for some hours in a micro flask, the reaction liquid being then distilled, the first drop being tested for boiling point, the second by the iodoform reaction. The weakness of the test as a general reaction is, of course, that not all sugars are attacked by ordinary brewers yeast (page 337).

MOLISCH'S TEST. The substance is treated with ten times its weight of an alcohol solution of alpha naphthol, and an equal volume of concentrated sulphuric acid. A deep violet is produced, but only by ready-formed sugar.

TABLE II

GENERAL SEPARATION SCHEME

Steam Distils	Does not steam Distil						
	Insoluble in Ether	Soluble in Ether					
		Soluble in Acid; Insoluble in Alkali	Soluble in Acid and in Alkali	Soluble in Alkali; Insoluble in Acid		Insoluble in Acid; Insoluble in Alkali	
		Basic	Amphoteric	ppd. by CO_2	Not ppd. by CO_2	Soluble in $NaHSO_3$	Insoluble in $NaHSO_3$
Hydrocarbons, ethers, alcohols, esters, ketones, aliphatic halogen compounds, and members of other classes with B.P. below 100° C.	Metallic salts, mineral acid salts, carbohydrates, amino acids, and sulphonic acids.	Aliphatic and aromatic amines; some amides.	Oximes, amino phenols, etc.	Phenols: Phenols, mercaptans, keto-enolic bodies.	Acids and Nitrophenols: Carboxylic acids, o. and p. nitrophenols.	Aldehydes and Ketones: Aldehydes and ketones and their substitution products.	Hydrocarbons, ethers, alcohols, esters, nitriles, sulphides, sulphonamides, mineral acid esters.

Aniline + Aniline HCl, heated with the substance: acrolein: yellow amorphous pp. of allylidene aniline. Anisaldehyde: like benzaldehyde. Benzaldehyde: (warmed) violet diamino triphenyl carbinol. (Note: with HAc in place of HCl, forms amorphous benzylidene aniline). Cinnamic aldehyde: yellow colour, later brown mass. Cuminol: green mass, and a small amount of a violet dye. Furfurol: bright blue condensation product.

Indophenol Reaction. Add p. amino dimethylaniline to a sodium carbonate solution of the hydroxy compound, add a little hydrogen peroxide, and stand ten minutes. Phenol: a blue indophenol.

Quinaldine Synthesis. Add to the aldehyde a little sulphuric acid, distil a quarter over, acidify with acetic acid, shake out with a few drops of aniline. A cloudiness indicates aldehyde. If no cloud appears shake out with petroleum ether, which extracts acetone, evaporate the extract to dryness, dissolve in four times the volume of concentrated HCl and boil for an hour. The ethylidene aniline splits, and condensation takes place to quinaldine. Evaporate, extract with water, add a dilute solution of potassium ferrocyanide + HCl to one half, and platinic chloride to the rest. Acetaldehyde: quinaldine formed. Formaldehyde: orange amorphous mass.

Thionine Dye Test. A drop of an aqueous solution of a suspected diamine is saturated with sulphuretted hydrogen, and a drop of ferric chloride is then run in. A blue coloration is given by diamines.

Indoaniline Test. Phenol is added to a dilute solution of the amine, followed by an excess of sodium carbonate; some hydrogen peroxide is then added, and the mixture allowed to stand until a blue cloud of the indoaniline makes its appearance.

Phthallic Anhydride Condensations. The reaction is carried out by heating the phenolic body to 200° C. for a few minutes in the presence of concentrated sulphuric acid, then dissolving the melt in water. The acid solution is decanted off, and the residue is dissolved in caustic soda. Only three compounds give fluorescent derivatives, the colour of fluorescence being as follows: Phenol: forms phenolphthallein. Resorcin: forms fluorescein (greenish-yellow). Hydroquinone: (blue fluorescence.) β naphthol: (blue-green fluorescence.

Quinoxaline Derivatives. A small quantity of the o. diamine is placed in a drop of dilute acetic acid, in an anilide tube, and heated almost to boiling. The solution should be quite clear. One drop of a solution of phenanthrene in sodium bisulphite, containing a little sodium acetate, is added. The derivative is separated as usual, and crystallized from hot water or aqueous alcohol. The crystalline form is often characteristic, and the melting point is important.

Benzoquinone (or alphanaphthoquinone) gives highly coloured quinhydrones. The ease of formation and crystallization decreases in the order para, meta, ortho. Heat should not be used in this reaction.

Separations. PHENOLS. The solubility in alkali is examined by the method on page 91. The solution obtained is filtered; one-half is deposited on a clean cover glass, the other on a clean slide. The cover glass is subjected to the action of HCl gas by method 107, the slide to an atmosphere of carbon dioxide by placing it over a small watch glass containing marble and *sulphuric* acid, the whole being covered by half a Petri dish. The drops are examined for solid insoluble in carbonate of soda, or in neutral salt solution.

Acid should not be added directly to the test drop, because many water soluble substances of the amine type are found to give a precipitate, whilst many amphoteric substances are only soluble in water over a limited pH range.

GENERAL SEPARATION SCHEME. Dissolve in about three times the amount of benzene, and add an equal volume of water. The mixture becomes milky on shaking, but the agitation should not be continued past this point. The monophenols and phenolic ethers dissolve in the benzene, the polyphenols being taken up by the water. After separating the two layers, the benzene is again shaken with water, which removes catechol and resorcin.

Monophenols. The benzene solution is steam distilled, or is distilled after adding a considerable excess of water. The distillate is collected two or three drops at a time; the fractions will contain the monophenolic bodies in the order of their boiling points. The drops may be evaporated to dryness, or may be extracted with a few drops of benzene.

Polyphenols. The aqueous extracts are warmed to free them from phenol and benzene; lead acetate is then added which precipitates catechol and pyrogallol. The precipitate is washed by decantation, decomposed by sulphuric acid, and carefully evaporated to dryness. The catechol is extracted with benzene, and the pyrogallol with alcohol.

Lead is removed from the original liquid by sulphuric acid, and after evaporation, resorcin is extracted with benzene.

Meta phenols may be precipitated as the tribromo compounds, avoiding excess of bromine. If excess is added, it may be removed by gently warming after the addition of formic acid.

Para dihydroxy bodies may be oxidized to quinhydrones by careful treatment with acid permanganate, or finally to quinones, the reactions being then more characteristic.

The entire group of phenols may be precipitated from its alkali salt solutions by the addition of an excess of ammonium carbonate, or by saturation with carbon dioxide.

Diazo sulphanilic acid produces colorations with all phenols, except in the rare cases where the substituting positions are blocked.

The picrate test for hydrocarbons is usually applicable to the naphthols.

All phenols sublime easily and completely. Steam distillation removes phenol, cresols, xylenols, thymol, and a small amount of naphthol.

Primary Amines. A general scheme for the separation of the more important groups of compounds might be arranged as follows—

1. Steam distil. NaOH may be added to fix phenolic and acidic bodies if thought necessary. Aniline, toluidines, and xylidines distil over. The residue is made up of naphthylamines, diamines, and the benzidine group.

2. Extract with benzine. Naphthylamines and cumidine are removed. The residue contains diamines and benzidines.

3. Add water, warm, cool, and filter. This removes the diamines. The benzidine group, which is insoluble in water, remains.

4. Extract with isobutyl alcohol, after adding an excess of potassium carbonate, if m. diamines are suspected. The diamines are present in the extract.

Each of these fractions may now be examined.

Aniline Group. During the steam distillation, the drops may be collected separately. The first to come over is aniline, next the toluidines, and last the xylidines. A very small amount of pseudo cumidine may come over, and will crystallize in the last drops.

For further separation, the insolubility of certain salts may be employed. An addition of sodium nitrate to an exact solution in dilute HCl precipitates meta xylidine and ortho toluidine. After filtering, sodium oxalate may be added, which precipitates para toluidine from fairly concentrated solution.

With care, a certain amount of separation may be obtained by fractional precipitation with potassium antimonyl tartrate, which precipitates ortho compounds first, then meta, and finally para. This reagent is thus useful in order to obtain some idea of the orientation of the groups, for the crystals differ, e.g.—

Ortho (and cumidine): spikes and needles, stars and fans.

Meta (especially after adding sodium tartrate to reduce the solubility): rhombic plates and groups.

Para (and aniline): thick hemihedric crystals (of the same type as those of the reagent).

Naphthylamine Group. The extract should be evaporated to dryness and extracted with petroleum ether, which removes the alpha amine and pseudocumidine. These are easily distinguished from each other by means of tetrachlorquinone.

The residue is beta naphthylamine.

Diamines. Ortho diamines are precipitated from weakly acid (HAc) solutions in presence of sodium acetate by means of alloxantin, Para diamines form insoluble sulphates, which slowly come down after the addition of sodium sulphate. The meta diamines are very soluble in water, and require the addition of potassium carbonate before extraction. Potassium ferrocyanide is the most useful reagent for the further testing of meta diamines.

Benzidine Group. Separation is difficult, fractional precipitation by means of caustic soda being the only microchemical method available. From a solution of the hydrochlorides, benzidine is precipitated instantly, dianisidine after a few seconds, but toluidine much later, often requiring several minutes to elapse. The solutions should not be too concentrated.

SECONDARY AMINES. Separation is difficult. Pyrrol is dissolved by dilute alkalis, whilst weak acids dissolve the commonly-occurring bodies belonging to this group except carbazole and diphenylamine. This residue may be examined by means of dinitroanthraquinone, which precipitates carbazole.

TERTIARY AMINES. Ammonium dichromate precipitates acridine and quinoline, and their homologues; the pyridine derivatives are more soluble. Pyridine bases may be obtained as iodoplatinates by first precipitating the quinoline group with platinic chloride in moderately concentrated solution, filtering, and adding sodium iodide to the filtrate. The quinoline group may be obtained by evaporating somewhat the mother liquor, and if necessary, when the pyridine homologue derivative is very soluble, precipitating as bromoaurate. The bases are obtained again by warming with NaOH and extracting with benzene.

The hydroxy and carboxy derivatives may be distinguished from the bases by means of tetrachlorquinone, as a group reagent.

A diazotized amine will couple with the hydroxy and amino derivatives but not with the unsubstituted bases. Mercuric chloride is useful for distinguishing between the pyridine bases, potassium ferrocyanide may be considered as the group reagent for quinoline bases, whilst tetrachlorquinone reacts with the hydro derivatives of both groups.

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CHAPTER IX

BOTANICAL TECHNIQUE

It is almost always necessary to give some kind of preliminary treatment to an organized vegetable structure before mounting it for examination under the microscope. This preliminary treatment has as its object either the concentration of impurities, if these are to be detected; the making of structures more obvious by methods such as section cutting, or by the disintegration of the structure into its constituent elements; by staining, or by dissolving out of certain types of structure. The methods employed may be divided into the five classes given below, and normally follow each other in this order. Industrial analytical work, however, often deals with substances which are in such a condition that they will not benefit by one, two, or more of these treatments, which are consequently omitted. Furthermore, the exact and lengthy methods which are necessary in the investigation of botanical structure are normally shortened a great deal in the works laboratory, since the shortened method, though not giving good mounts, provides the information required.

FIXING AND HARDENING. Methods of killing the cells and hardening the structure to prevent subsequent distortion.

PURELY MECHANICAL METHODS. These separate structures physically, and may be illustrated by section cutting.

MACERATION. Chemical methods which are much more vigorous than the preceding, and result usually in the destruction of the easily attacked tissues, leaving the more resistant parts behind.

STAINING METHODS. These methods colour some elements or impurities, but not the substance itself, or *vice versa.*

MOUNTING. Immersion of the preparation in a substance of such a refractive index that the optical properties of the objectives are fully employed, and the structure of the substance observed accurately.

Fixing and hardening. In a very large number of cases, the first operation to be performed is that of killing the organism or the cells of the structure. This is commonly known as fixing, and is usually accompanied by a process of hardening of the tissues, which serves to preserve the form during succeeding manipulations, such as, for example, clearing, or section cutting; and also prevents, to a greater or lesser extent, deformation by post mortem changes.

The properties of the ideal fixative are many, and never realized in one chemical; most fixative solutions are therefore mixtures, and their number is legion. The desirable properties are summarized below.

1. The tissues should not become swollen or shrunk by the action of the fixative. Few fixatives are without any action of this kind, particularly after long treatment, and even a mild fixing agent, such as 5 per cent acetic acid, may swell tissues considerably after a while.

2. Good penetration is desirable, and many otherwise excellent fixatives, such as osmic acid, have their use somewhat limited by reason of their superficial action. Unfortunately, the chemicals which penetrate best have often undesirable properties in other directions; picric acid, for example, which is excellent for penetration, has a negligible hardening action.

3. Cell contents should be made insoluble, in order to resist wet processes which follow, such as staining. It is often necessary, when staining after using such a fixative as picric acid, which does not affect cell contents, to avoid any aqueous solutions. Chromic acid is a typical fixative of the opposite type, making cell contents insoluble.

4. The hardening should not be so severe as to make the tissues brittle. Considerable variation is to be found amongst fixatives in this respect, potassium bichromate being one of the best.

5. Fixing is usually, if not invariably, accompanied by a raising of the refractive index of the tissue, and as this is not in the same proportion for all the tissues present, a certain amount of visual differentiation of structures is produced, making examination considerably easier. With some fixatives, such as osmic acid, this may easily be carried too far, the result being a highly refractive preparation of little use even when mounted in a special medium with an appropriate refractive index.

6. The fixing agent should not interfere with subsequent staining. Mercury salts are bad in this respect, and must be washed very thoroughly out of the preparation.

7. No coloration should take place during fixing. Osmic acid often colours certain cell contents very deeply, but the colours may be bleached by means of hydrogen peroxide.

8. It almost goes without saying that the reagent should have good keeping properties.

The time of action and strength of solution required for fixing vary enormously, and it is almost impossible to lay down general rules. It may be said, however, that for weak reagents, a considerable volume should be used; the time of action should be as short as possible, unless a large change in refractive index is required; and the washing should be very thorough, using either water or alcohol, as the case requires. Fixing solutions are most efficient when acid in reaction, and from 2 to 5 per cent of acetic acid should normally be added.

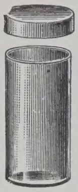
Fig. 113. Specimen Jar

After fixing, objects which are to be kept for some time may be preserved in alcohol.

Picric Acid. A saturated solution is used in either 95 per cent alcohol or water; for general purposes it is excellent, being easy to use and reliable. Cell contents, however, are not fixed, and alcoholic stains should be employed, unless a fixing or hardening agent for cell contents forms part of the stain. It penetrates very well, and is suitable for thick sections or other thick objects. Several hours fixing is required, and the acid should afterwards be washed out with alcohol. (No 29.)

Osmic Acid OsO_4. This is an old fixative, but well worth using in conjunction with other fixing agents. One of the best of these mixtures, especially for delicate sections and tissues, is due to Flemming. (No. 31.)

Osmic acid alone may also be used, in the shape of a 1 per cent solution, which, however, does not keep at all well unless traces of an oxidizing agent, such as chromic acid, hydrogen peroxide, or potassium permangate, are added at intervals. It should be mentioned that osmic acid is a powerful oxidizing agent.

The fixing with osmic acid alone may be carried out by placing the slide to which the object is attached (in the case of a section) face downwards over a wide-mouthed bottle containing the solution. The time of action may vary from 1 min., for delicate preparations, to many hours. This method is to be recommended, as it entails much less washing than the immersion method.

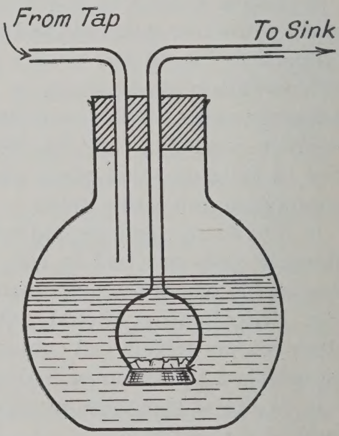

Fig. 114. Washing Flask

The object may, on the other hand, be steeped in the osmic acid solution, or Flemming's solution, but very thorough washing is required, from 4–6 hours for small objects up to 24 hours for large ones, the water being frequently changed. This is followed, if necessary, by an immersion in dilute hydrogen peroxide in order to bleach it, especially when fatty cell contents are present, as these are stained almost black.

It should be noted that osmic acid is very irritating, and very readily causes catarrh and painful eye diseases.

Osmic acid fixative solutions are of value mainly for thin tissues or sections, as penetration is bad. Over fixing is also to be guarded against, as it results in loss of detail.

CHROMIC ACID. A 1 per cent solution in water is employed, with the addition of a little acetic acid (1 cc. per 100). It has a tendency to make tissues brittle, and unless well washed out may interfere with staining. Cell contents are well fixed, however, and water stains may be used subsequently. It is an excellent fixative for general use, but may require to be diluted for delicate objects.

Potassium bichromate is often employed, from 2 to 5 per cent in strength. Washing must be carried out thoroughly. Hardening is slower than with chromic acid, and there is much less danger of the structures becoming brittle.

Should the preparation become coloured objectionably by the bichromate, it may be bleached by sulphur dioxide solution.

MERCURIC CHLORIDE. For general work this fixative can be recommended, a saturated solution in 1 per cent acetic acid being effective. Washing must be carried out very thoroughly with alcohol-water 3 : 1.

A variation of this fixative often of value, is prepared by adding picric acid. (No. 30.) Flatters states that this is the most complete fixative known, but it darkens many cell contents, especially of the resin type.

Section Cutting. A cylinder may be cut in section in a variety of ways.

TRANSVERSE SECTION. Cut in a plane at right angles to the long axis.

CONIC SECTION. Cut in a plane making an angle other than 90° or 180° with the longitudinal axis.

RADIAL SECTION. Cut longitudinally in the plane of the long axis, the section thus lying along a radius.

TANGENTIAL SECTION. Cut in a plane parallel to the long axis, usually near to the circumference. The plane of cutting is thus at right angles to some one radius, and is parallel to a radial section.

The appearance of these various sections differs greatly. The transverse section, in the case of a lead pencil, for example, will show two concentric circles; the conic section will exhibit two ovals;

whilst the radial and tangential sections will take the form of double rectangles.

A more complex structure, such as a cart wheel, in a radial section would give a longitudinal section of the hub and two of the spokes, but a tangential section would show several of the spokes in conic section.

It is evident, therefore, that great care must be taken in both the cutting and interpretation of the cross-sections of organized structures. The transverse and radial sections are easiest to interpret; the tangential section is more difficult, whilst the conic section is rarely of value.

The method of section cutting employed will depend upon the substance under examination. For rough work, or for soft substances, hand-cutting may be used, but when very thin sections are required, or when the physical condition of the substance makes it difficult to obtain suitable sections by hand, one of various machines must be employed.

The substance is ideally soft and waxy, but in the majority of cases it will be too hard; only occasionally will it be found too soft. Some method of embedding the object is therefore almost always necessary, which may vary from a simple embedding in pith or cork, to an impregnation with wax, or with collodion.

Pith Embedding. Pith may be obtained from most watchmakers. A piece rather thicker than a pencil, and about an inch long, is cut lengthwise along the centre; a V-shaped channel is then cut down the centre of the flat surface of each half, in order that the object may be gripped firmly. The object is placed within the two halves of the pith in such a position that the pith projects past the end of the object to some extent. The combination is then bound round with tape, care being taken that the tape is not carried to a point where it will interfere with the cutting. Pith and cork embedding are only used for hand-cutting.

Hand-cutting by Razor of a specimen embedded in pith.

The embedded specimen is held vertically between the (horizontal) first finger and thumb of the left hand, the top of the pith being slightly above the level of the first finger, the thumb being well down out of the way of the knife. The razor used for cutting should be flat ground. The flat side is rested on the forefinger of the left hand, with the edge towards the worker, and the tip of the blade (farthest from the handle) just touching the pith. By pushing the razor from right to left, and at the same time pressing it forwards gently but firmly, a section will be cut. The razor blade should be as sharp as possible, and is often moistened with alcohol to advantage. The sections should be cut very thin, and transferred to water by means of a brush.

Cork Embedding. Pith is always employed, except in one special method of cutting sections of certain textile fibres. A finely-grained cork, $\frac{3}{8}$ in. diameter, is cut in two at right angles to its axis. A fine sewing-machine needle threaded with a length of thin but strong thread, is pushed through the cork, then slightly withdrawn, leaving a loop of thread projecting. Inside this loop is placed a bundle of textile fibres (artificial silk, for example), which when doubled, is rather more than the diameter of the needle. The needle and loop are together pulled out of the cork, leaving the textile fibre tightly embedded. Both ends of the cork are then trimmed with a knife, making them as parallel as possible. The special features of this method is that a thin slice is now taken off *both* ends of the cork, leaving a length of fibre of, perhaps, 3 or 4 mm. (The usual section is of course cut as thin as possible.) If the fibre under examination is transparent, such as viscose silk, this thick section may be fixed on a slide by means of a spot of plasticine at one edge, and viewed as usual by transmitted parallel light; the light is conducted inside the fibre by internal reflection. For such fibres the method, which is due to Herzog, is extraordinarily good. More opaque fibres of complex structure, or such artificial silks as have air bubbles in their interior, must be viewed by reflected light, and the method, though useful for rough work, is not too satisfactory.

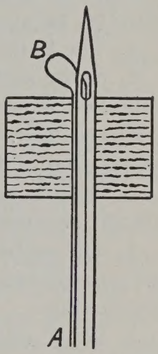

FIG. 115. NEEDLE AND THREAD METHOD OF EMBEDDING FIBRES

A. Loop of thread.
B. Thread continuing through the cork.

The two most important remaining processes are the *wax* and the *collodion* methods, so named because of the embedding material employed. The old-established wax method was at one time thought to be in danger of being superseded by the collodion process, but at the present time, in the writer's opinion, for general analytical work the wax method is by far the best, except in special cases, and should always be tried before resorting to collodion embedding.

The relative advantages of the two methods may be summarized as follows—

PARAFFIN WAX. 1. Thinner sections may be cut, except in the case of objects more than 2 cm. or 3 cm. square.

2. The relative consistency of the specimen and embedding medium plays an important part in the cutting of good sections; therefore, in analytical work, in which much variation in the

hardness of the objects examined occurs, the wax method is more convenient, because wax slowly hardens during some hours after setting, and will at some time pass through the best condition for cutting, this being found by occasional trials at intervals.

3. The wax process is much more rapid than the best collodion method.

4. The embedding material is completely removed before staining and mounting, with the result that no trouble is experienced by the matrix being stained; this often happens with collodion embedded objects, because the collodion is not removed.

5. The knife need not be kept wet during cutting.

6. Serial sections are cut with very much less trouble than when collodion is used.

COLLODION. 1. Brittle and crumbly objects are held together better than with wax.

2. Objects need not be cleared before embedding (this, though not always essential, is often advisable in the wax process).

3. The embedding mass is transparent, enabling the object to be very easily orientated in the microtome cup in the desired position for cutting.

4. Large objects may be steeped in the embedding medium for weeks without injury, to ensure thorough impregnation; such a prolonged steeping would be ruinous in melted paraffin, owing to the temperature employed, which, to use Dr. Lee's expression, would "cook" the specimen.

5. For routine examinations of one class of substance, the hardness of the embedding medium may be exactly controlled and reproduced to suit the object in question, by the addition of so-called plasticisers, such as triphenyl phosphate, to the collodion.

6. It is unnecessary to remove the collodion before staining and mounting.

In certain instances the two methods may be combined, the object being first treated lightly with collodion and afterwards embedded in wax for cutting.

Wax embedding. The melting point of the wax employed will depend upon the normal temperature of the laboratory. For cold rooms, a low melting-point paraffin wax is required, about 45° C. In normally heated rooms a wax melting at about 55° C. will be suitable; whilst for use in a warm laboratory, a filtered beeswax (filtered through a hot water funnel in order to remove pollen grains) will prove more satisfactory. A harder wax is required in summer than in winter; rocker microtomes require a harder wax than other types.

The steps in the embedding process vary according to circumstances, and the following rather lengthy method can often be somewhat simplified.

CLEARING. The object well dehydrated in the usual way, is soaked in some clearing agent, which is a paraffin solvent. Such reagents are *chloroform,* which is suitable for small objects only; *aniline,* which is useful for damp substances; *cedar-wood oil,* which is very serviceable, as it penetrates well, does not make structures brittle, and if reasonably well removed does not affect the cutting of the wax; *turpentine,* which is more suitable for beeswax than for paraffin; *xylol,* which may be recommended for general use as being the most convenient, and has several advantages over chloroform; the preparation should be well dehydrated

FIRST IMPREGNATION. If a volatile chemical, such as xylene or chloroform, is being used, the clearer, containing the specimen, is raised to the melting point of the wax, some molten wax being then poured in almost to saturation. The solution of wax is then allowed to evaporate completely, every trace of the solvent being carefully removed, or the wax will cut too soft. If the block of paraffin is milky in appearance, the object may not have been thoroughly dehydrated; or chloroform (if employed) may not have been completely driven off.

When cedar-wood oil or clove oil is used for clearing, the drained object is placed in a bath of melted paraffin for some time, until it is judged to be impregnated. Rather large objects carry over a comparatively large amount of clearing agent; in this case the bath may require to be changed once or twice, which removes traces of the clearing agent.

FINAL IMPREGNATION. The object, treated as indicated, is placed in a bath of melted wax. It is important that the wax should not be heated more than a degree or so above its melting point, as with continual heating at high temperatures, the melting point rises; for this reason the object should be kept in the wax only sufficiently long to ensure complete impregnation, two hours being ample.

Objects which are difficult to impregnate may be embedded in vacuo.

Very small objects may be tinted in the preliminary bath by adding a little colouring matter to the wax (they are not then so liable to get lost in the final block of wax); rather larger specimens may be embedded in wax contained in a deep watch glass; still larger ones may be put into melted wax contained in a cardboard pill box.

It is useful to wipe glass or paper containers with a rag moistened with glycerine, as the wax then comes away very easily.

Textile fibres may be wrapped round a wire frame as sketched; after a preliminary soaking in melted wax, the frame is placed in a brass tube, which has a screw adjustment. Wax contracts considerably on cooling, particularly if initially a few degrees above its melting point, and unless a screw adjustment is used, a crack develops along the axis of the wax cylinder, causing the fibres to be only loosely embedded. The screw is turned slowly whilst the wax is setting, at a rate which keeps the wax flush with the top of the tube. As soon as the wax sets, the pencil begins to move out, and may then easily be removed when cold. Instead of such a tube, a test tube may be employed, but in this case 2 in. of wax is necessary above the fibres.

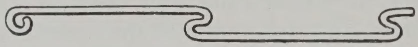

Fig. 116. Wire Frame for Embedding Textile Fibres in Wax

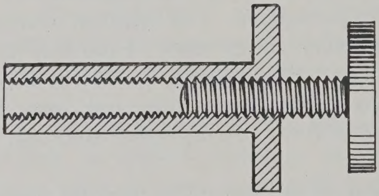

Fig. 117. Brass Tube for Wax Embedding of Textile Fibres

Rapid cooling of the wax is advisable, as this minimizes crystallization, which always sets in to some extent, and interferes with the cutting of very fine structures.

Semi-mechanical Cutting. When thick sections only are required, of wax-embedded masses, the Cole microtome (Fig. 118) may be used. The knife slides along the flat upper surface of the microtome, the wax pencil being raised slightly by means of a screw piston after each section is cut. Other models of similar construction are due to Flatters and to Watson.

Mechanical Cutting—The Cambridge Rocker Microtome. The object, embedded in wax, is fixed in the cup of the microtome by means of molten wax. Before the wax mass is so fixed, it must be cut to a suitable shape, this depending upon the cutting angle of the knife, and whether the sections are to be cut in ribbons or not.

The notes given here apply also, in most instances, to collodion embedded objects, with the exception that it is unnecessary to shape the embedding mass.

The *slope* of the knife, i.e. the angle made by the flat of the knife with the plane in which the object is being cut, varies to some extent with the object, and also with the way in which the razor is ground. For general work, a very slightly hollow-ground razor is quite suitable, and this should be set at an angle of about 10° with the cut surface of the wax; the thick edge of the knife should, of course, slope away from the wax. When ribbons are being cut, this angle

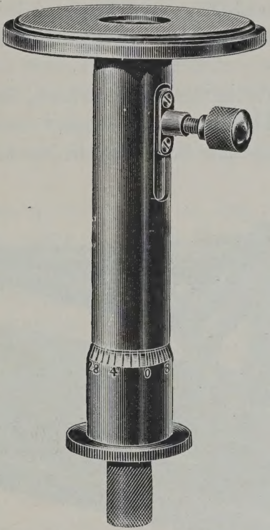

Fig. 118. Cole Microtome

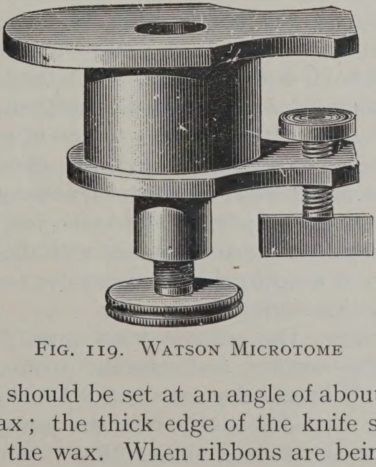

Fig. 119. Watson Microtome

should be reduced; very hard objects, or sections which tend to be brittle, are also best cut with a knife which merely clears the cutting plane in its thick part. If a straight ground razor is being used (there is, however, little advantage to be gained by the use of this form of knife in mechanical cutting) the tilt, of course, may be a little less.

The *slant* of the knife is the angle which the cutting edge makes with the horizontal. The larger the object, or the more complex its structure, with regard to the variation in hardness of the constituent elements, the more the knife should be slanted from the horizontal. It is sometimes stated that this slant results in the motion of hand-cutting, in which the razor edge is used as a very fine saw, being imitated, but this is far from the case. With the Cambridge instrument it is impossible, whatever the knife position, to do other than "chop" the section off. It is difficult to see why the slant of the knife should influence the cutting of sections, since once the cutting has begun, there is no difference whatever between this cutting at an angle, and the cutting with a horizontal knife, but there is no doubt that in practice better results are obtained with the class of specimen mentioned. There is a noticeable tendency for the sections to roll more in cutting with a slanted knife.

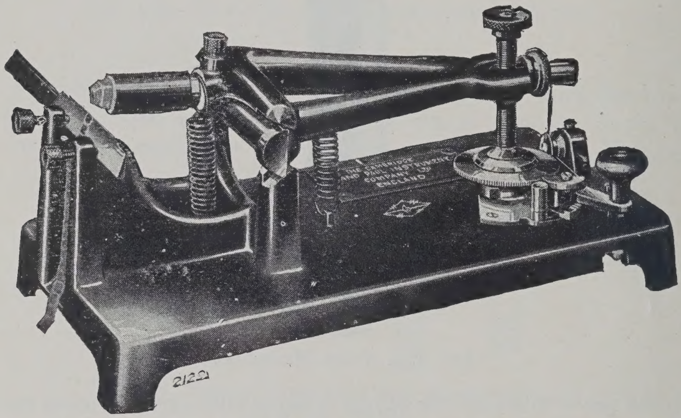

FIG. 120. CAMBRIDGE ROCKER MICROTOME

SHAPE OF SECTION. The cutting mass must be shaped to an exactly square cross-section, and carefully arranged in the cup so that the upper and lower edges are parallel and horizontal, when ribbons are to be cut. For single sections, however, it is always advisable to cut the mass to a cross-section which ensures that the knife enters by one point and leaves by another. When using a horizontal knife, the mass may be cut to a square cross-section, as for ribbons, but set with its sides at 45° to the knife edge. When the knife is used at a slant, the mass should be set in the cup in such a way that the sides of the square make an angle of 45° with the edge of the knife. It is sometimes recommended, when cutting by means of a slanted knife, to trim the mass to a triangle, and to set it so that the knife enters by one point and leaves it by another, as already stated. The value of this refinement is, however, somewhat doubtful.

A most necessary precaution is to set the object in the mass so that it is nearer to the point at which the knife leaves than to the point where it enters. There are two reasons for this, the more important one being that if the section curls in cutting, the object is on the outside (and flatter) part of the curl. The other reason is that the shock of the knife striking the embedding mass, causes an irregularity of cutting, which results in slight waves or irregularity in thickness in the first one or two millimetres; this may be seen under favourable conditions by examining a wax section with very oblique reflected lighting, before the wax is removed.

SPEED OF CUTTING. There is a peculiar knack in the working of a Cambridge instrument, which is impossible to describe, but which, to some extent, depends upon the speed at which the handle is pulled and allowed to go back. The more difficult the object, the slower should be the speed, as a rule, the exception being when ribbons are in question. In this case, a rather more rapid speed should be used; it is also very important to maintain a steady rhythmic swing, and, once the cutting has been commenced, the whole of the sections required should be cut without variation in speed. It is also bad practice to interrupt the cutting before the number of cuts wanted have been obtained. The knack is one which only comes by practice, and, unfortunately, is easily lost if one is not frequently cutting sections. These remarks apply, however, only to the cutting of difficult objects; satisfactory sections of the average specimen may be cut without much difficulty.

A few of the faults often met with may now be indicated, together with the means for their prevention, and cure.

ROLLING. This may be caused by an excessive slope of the knife. Wax which is too hard will also produce this fault; a lighted electric globe placed a couple of feet or so away from the microtome for a few minutes will quickly rectify this condition; indeed, an astonishingly small temperature difference is sufficient to alter cutting conditions considerably. A tilted knife always tends to cause rolling.

If the rolling proves very obstinate, the sections may be cut in the form of a ribbon; the attachment of the sections to each other diminishes the rolling greatly. A further method of overcoming the difficulty is to cut the embedding mass so that it is four or five times as long as necessary, arranging the object as far away from the knife as is practicable. The section thus cut is on the outside, and almost flat, portion of the roll.

Curled sections may be straightened out by floating them on water in a small dish, and gently warming the water, or they may be flattened by gently warming them after they have been floated on to the slide.

CRUMPLING. The paraffin may be too soft, in which case a small piece of ice, or a beaker containing a freezing mixture, may be placed near. If the knife is too blunt, this fault will also occur. It may be remarked here that the knife cannot be obtained too sharp by ordinary stropping and honing methods.

RIFTS. The wax section as a whole may show broken furrows, which are due to the knife being set at too great a slope. A very brittle object may break during cutting, though the wax does not, in which case the exposed surface of the mass, after each cut, may be lightly brushed over with an almost dry brush dipped in collodion solution. The edges of the mass must be left untouched. If the wax tears round the section, the relative hardness of the wax and object requires adjusting, and a harder wax is necessary. A slight nick in the knife, or a small fragment of adherent solid on the knife edge, may also cause split sections.

FIXING ON SLIDE. Sections must be fixed by some means to the slide, sufficiently firmly to ensure their being held in position there during the dissolving out of the wax. This is most easily done by rubbing on the slide a 1 per cent solution of a good gelatine, until the film is barely visible. A better solution for the purpose is Mayer's albumen (90), though it is rather tedious to prepare, and does not keep very well, even a month causing considerable deterioration.

The sections are floated on water in a fairly large dish, and the treated slide is brought underneath them, under the water. By this means the most delicate of sections may be transferred to the slide without damage. The water is allowed to drain off, and any slight crumples or curls are taken out by very cautious warming. The flattened sections are left in position until the slide is dry; drying may be accelerated by gentle heat, such as is provided by an incubator for bacteria. When dry, the preparation is heated barely sufficiently to melt the paraffin. The slide is then plunged into xylol which further coagulates the albumen, and at the same time removes the wax, leaving the sections glued on to the slide, and free from wax. When beeswax is employed, a slight residue is often left, which may be removed by means of turpentine if desired; in certain cases where its presence is not objectionable, it may be left, since it almost completely disappears on mounting in Canada balsam.

For most routine work the preparation is rinsed with alcohol once or twice, stained if required, and mounted for examination. It is advisable, after transferring the section to the clearing agent, to examine under dark-ground illumination. A milky appearance indicates incomplete dealcoholization, and the clearing should be repeated.

The preparation, before staining, should be graduated through xylol + alcohol and alcohol + water mixtures to an alcoholic concentration, of the same strength as the staining solution, for very accurate work. After staining, the usual dehydration and clearing process is followed for mounting in Canada balsam.

THE SPENCER MICROTOME. This instrument, which cuts plane surfaces (in contrast to the Cambridge, which cuts very slightly-curved sections) is very rapid in action, and is particularly suitable for collodion section cutting. It is, however, rather expensive compared with other forms of apparatus.

Collodion embedding. There is an essential difference between the paraffin and the collodion methods of embedding, in that it is not necessary to remove the collodion before staining and mounting. The relative advantages and disadvantages of the two methods have already been discussed.

The object must first be thoroughly dehydrated with alcohol, after which it is soaked in ether for some hours. During this time, the solution of collodion may be made by cutting the perfectly dry collodion (Schlering's celluloid chips) into thin chips, and allowing them to steep in a mixture of alcohol and ether. The collodion at first swells and becomes gelatinous, finally dissolving to a thick viscous solution, which should have a concentration of about 1 : 15. In this bath the object must be soaked for some days, and as it is upon this first soaking that successful sections depend, the steeping must not be hurried. There are, however, simple objects which are to be examined as a matter of routine for their more obvious details, which may be steeped for an hour or so only: such objects are the artificial silks.

When the specimen has been thoroughly infiltrated in the first bath, it should be transferred to a second and stronger solution, about 1 : 10, which, in the case of small objects, may be in a small pill box of ½ in. diameter. The object may be orientated in this bath by means of needles wet with the alcohol-ether mixture. By evaporation the collodion shrinks until the object begins to appear above the surface. More collodion must now be poured in and the evaporation continued. It may be necessary to repeat this operation once or twice until the mass is so hard after evaporation that no impression is made by the finger. The side of the pill box is now torn off, and the mass coagulated by immersing it in chloroform for a few hours; 85 per cent alcohol may be used to replace the chloroform. When time is not very important, better hardening is obtained by means of chloroform vapour, the operation being carried out by placing the embedding mass in a beaker, which contains a small crucible in the bottom, filled with chloroform.

A much more rapid and very serviceable method of embedding is due to Gibson. The object is transferred from the ether bath into a thin solution of collodion in a test tube, which is placed in a paraffin bath (not a water bath) at such a temperature that the solvent boils freely. This boiling does not damage the specimen, because it takes place at a low temperature. When the volume has decreased to about a third of its original capacity, the syrupy mass is turned out and mounted on a block of collodion which has previously been hardened in chloroform. The whole may now be hardened by one of the methods previously given, an excellent modification when time presses being a mixture of chloroform and cedar-wood oil, which clears as it hardens.

Cutting of Embedded Specimens. The object having been embedded by one of the preceding methods, it must be fixed in the microtome, by a method appropriate to the instrument employed. For the purposes of general analytical work, it is necessary to be familiar with the types mentioned on pages 175, 176, and 179.

COLLODION EMBEDDED MASSES—CUTTING PROCESS. The hardened mass, by whatever method prepared, is fixed in the cup of the microtome either by a mixture of beeswax and resin, in the proportion of two of the latter to one of the former, or by collodion.

The knife during cutting, each freshly-cut surface, and the top of the block, must be kept moist with cedar-wood oil, or with 85 per cent alcohol. If the latter is used, the concentration mentioned must be adhered to for good results.

The sections as cut are transferred to alcohol, and from thence to the slide. They are fixed to the slide by exposure to the vapour of a mixture of alcohol and ether for some time. The slide must be perfectly clean and free from grease.

The sections, once fixed to the slide, may be stained without removing the collodion, as the embedding medium either does not stain, or stains so weakly with the majority of the important stains, that the colour is easily removed by alcohol.

Mounting may be done in either glycerine jelly or Canada balsam, without removal of the collodion, the only precaution necessary in the latter case, being to clear with an agent, such as cedar-wood oil, xylol, or oil of bergamot, which does not dissolve the collodion.

MACERATION. This term covers a number of very diversely conducted operations which yet have this in common, that they result in the simplification of the structure which is being examined, either by removal of certain constituents, or by separation of a complex structure into its simpler elements. The objects to be attained are summarized briefly below, the particular process employed depending upon the complexity of the structure and the degree of resistance to reagents which is expected.

1. In some cases a mere swelling takes place, which brings out the structure, as when a cotton hair is treated in Schweizer's reagent, to bring out and make evident the cuticle.

2. A separation may be effected into component cells, by the simple solution of the adhesive matter, as in the treatment of wood with nitrochromic acid.

3. A more severe action results in the destruction of certain cell constituents, or further action may dissolve out some constituent cells completely, as in the preparation of crude fibre.

4. The severest treatments leave only the most resistent matter as a residue, as in the wet ash process.

The end point is found in all cases by taking a little of the treated substance and testing it by mounting on a slide. The structural elements are readily separated by sliding the cover glass in a circular manner with gentle pressure.

Water. Simple exposure to moisture is sufficient to soften almost all leaves and many parenchymatous roots and rhizomes.

Soaking in water, often for more than a week, is necessary for many roots and rhizomes, and all woods and barks. Soluble cell contents tend to be removed; many insoluble contents swell considerably, and distortion usually occurs because the rates of swelling of various constituent elements are not the same.

Boiling water causes a softening of more resistent dried tissues, such as woods, and a swelling to approximately the original size. From 15 min. to half an hour is usually adequate. The action is aided by the addition of a little ammonia to the water, or even KOH.

When a dried specimen has become discoloured, bleaching may be resorted to after softening, by means of a dilute solution of sodium hypochlorite.

Water may also be employed in special cases as a solvent, for example, in the examination of sugars for an insoluble residue which may contain sugar mites, fragments of wood, and dirt due to careless handling.

Fat solvents. $CHCl_3$ CCl_4 ether, benzene, etc., may be brought into service, mainly in such cases as the examination of lubricants or ointments.

Alkalis. 2–5 PER CENT CAUSTIC SODA. The action is in the first place one of swelling, only the most resistant tissues being unaffected to some extent in this way. Following this, comes a solvent action, more particularly for starch and proteid cell contents, and the softer parts of insects. Finally, there is found a certain disintegrating action on cellulosic structures.

The more resistant structures are less readily attacked, such tissues being lignified and cuticularized walls, fibre, sclerenchymatous structures, and the chitinous parts of insects. In many instances these may be isolated very completely, and are thus more readily examined.

The most usual procedure is to cut up the object into small pieces, and boil in a small conical flask with the alkali until the action has proceeded sufficiently far. This may be proved by taking out a small portion, and teasing it out on a slide for rough examination. The substance should be washed by decantation several times with distilled water, then with a very weak acid, and again with water. A mineral acid should not be used for the purpose of neutralization, as, if it is not completely washed out, much damage is done to vegetable matter on drying.

A 5 per cent solution may be used for such purposes as the removal of protein matter from tinned fish, the characteristically-shaped fish scales being undissolved. Two per cent caustic will not dissolve the chitinous parts of mites, but is sufficiently strong to remove horse hairs, etc., thus enabling parasitic mites to be more easily examined, such as mange mites.

Ten per cent alkali, used boiling, provides one of the best methods for separating animal fibres from vegetable fibres in textile work.

Fifty per cent KOH is also often used, when a more severe action is required.

Acids. HYDROFLUORIC ACID. (Commercial.) (*N.B.*—This acid must not be allowed to touch the skin. Although not painful at the time of contact, a day or two later unpleasant and slow healing sores develop.)

Treatment with this acid may be carried out conveniently in a Petri dish which has been painted inside with wax. Its chief action is the solution of mineral matter, and the softening of hard tissues. It is a very slow maceration reagent, but its action is excellent for difficult objects, such as nut-shells, stones of fruit, and harder woods, especially when sections are to be cut.

The specimen should be well boiled in water, and after cooling left in the acid, until it will cut easily with a knife. This may take as long as six weeks. It is then washed very thoroughly with water, and placed in a mixture of equal parts of alcohol, glycerine, and water for a week, after which it is ready for cutting. A 20 per cent acid may also be used to extract starch from powders.

Hydrochloric Acid (20 per cent strength). A boiling solution removes starch, and the reagent is an excellent means of obtaining mites from the sifted residue from flours. After treatment, centrifuge, wash with alkali to neutralize, then with water, and mount the mites for examination.

Nitric Acid (20 per cent). This is useful in the treatment of many botanical structures, notably in the removal of the epidermis from leaves, and in cases where the swelling action of caustic alkali is objectionable. It is used boiling, and after half an hour at the boil, the specimen may be allowed to steep for two or three hours in the hot solution; a thorough washing must follow.

Chromic Acid. A concentrated solution was formerly used, cold, from 1 to 5 min., this being followed by a thorough washing, but it is now replaced for many purposes by Schultze's method.

When mixed with nitric or sulphuric acid, however (equal volumes of the 10 per cent solutions of the acids being taken), it is still very serviceable, as small portions of the specimen may be mounted in the reagent, allowed to stand for a few minutes, and broken up on the slide by moving the cover glass about. This method is very convenient, and should always be tried before proceeding to more elaborate ones. The elements of sections are often separated, the original disposition being retained.

Schultz's Method. This is one of the most often employed processes for botanical work, particularly for very resistant structures. Much quicker than the HF method, the results are little inferior, and when sections are not required, it is on the whole to be preferred.

Half an inch of concentrated nitric acid (60 per cent acid is often recommended) is placed in a test tube, and the material, first cut up into small pieces, is added. After the mixture has been boiled for a minute or so, add a little $KClO_3$, again warm, and add $KClO_3$ until bubbles are freely formed. Continue the action until the material is quite white, when the contents of the test tube are poured into a small beaker of water, and washed well by decantation. The separation of the tissues may be carried out by adding the fragments of macerated substance to a little water in a test tube, and shaking well, but, as a rule, the only further treatment necessary to separate the constituent tissues, is to mount and move the cover glass gently. Only in rare cases is it necessary to tease the substance out. Woods are first darkened, but later become bleached, and finally are disintegrated. Prolonged action results in total destruction of the cells.

Sulphuric acid. WET ASH. This drastic treatment has a limited application in the isolation of siliceous skeletons or diatoms.

Organic matter is destroyed by boiling with 20 per cent HCl, after which the solution is filtered on filter paper in a Gooch crucible. Wash well, drain by suction as much as possible, and drop, together with the filter paper, into 20 cc. of concentrated sulphuric acid in a hard glass flask. The sulphuric acid is heated on a sand bath as in the determination of nitrogen by Kjeldahl, until the acid is colourless. This may be hastened by the addition of a fairly considerable amount of dry potassium sulphate, or the cautious addition of 100 volumes hydrogen peroxide, a drop at a time.

The remaining operations consist of cooling, diluting with water, settling in a settling tube (this is preferable to centrifuging, as the structures are not so liable to damage), and mounting. For permanent mounts, dammar is better than Canada balsam, but for observation purposes Canada balsam in xylol, or better, in turpentine, is suitable.

Ten per cent acid is, of course, employed as a test for $CaCO_3$ in plant structures, characteristic crystals of $CaSO_4$ being formed. More dilute solutions may be employed to hydrolyse carbohydrates and derived substances, such as gums, the resulting sugars being often useful aids to identification.

Eighty per cent acid dissolves all unsuberized cellulosic structures, leaving behind the cuticle and the other suberized parts of the substance.

Crude fibre. A quantitative determination of so-called crude fibre is often made; the process consists in isolating the more resistant portions of the substance, more especially grain and flour, by treatment alternately with boiling acid and alkali. The latter removes proteids and colouring matter, the former dissolves out starch. The method is of great importance in dealing with starchy materials and highly-coloured substances.

Two processes are in general use: the Dutch, which is rapid, but employs concentrated solutions, and the American, which takes much longer but is more manageable.

American Method. Two gm. powder is boiled with 125 cc. of 2 per cent sulphuric acid for half an hour. Strain through a fine cloth, and wash on a Buchner funnel, then boil in 125 cc. of 2 per cent NaOH for half an hour, again strain and wash, and if thought necessary, repeat the acid process.

Dutch Method. Two gm. powder is boiled with 50 cc. of 10 per cent nitric acid for thirty seconds, filtered at the pump on cloth, washed well with boiling water, boiled for thirty seconds with 50 cc. of 2·5 per cent NaOH, filtered, washed, and mounted.

The substance is scraped off the cloth used for filtering by stretching the cloth over a watch glass until it is taut. The residual crude fibre may be preserved for reference in a dilute solution of a hardening agent.

Staining. The factors which decide the stain employed, and the method adopted, are many and varied. Each worker has his own preferred process of fixing, preparation, and mounting; in addition, certain classes of objects have been found to respond best to definite routines. The result is that the literature abounds with strongly-recommended methods and stains, each excellent for its own purpose; few of these are of general application. The methods indicated in the following pages will almost certainly require to be modified in detail to adapt them to specific analytical work, but they are put forward as a general introduction to the subject, and will enable satisfactory results to be obtained in most instances.

Staining may be carried out at almost any stage in the preparation of a substance for examination, but it is usually most satisfactory to stain after cutting sections, or after maceration.

Botanical cross-sections should be freed from chlorophyll before cutting, by steeping in methylated spirits, changed daily until colourless. If necessary, they may be bleached by means of a cold 5 per cent solution of NaOCl, taking care to wash very thoroughly afterwards.

The treatment of the section after cutting and before staining depends upon the method employed for cross-sectioning, and notes on this point will be found under the proper heading.

Fibres or powdered substances may be stained without any special preliminary treatment.

Staining operations may be divided into two main classes, general, and selective. The former class stain the majority of vegetable structures to a greater or less extent, according to the affinity of the structure for the stain. The result of such staining is to accentuate the optical differentiation which is already present in a properly fixed specimen, and to render detail more easily seen in consequence.

Selective stains, however, exhibit a strong affinity for structures of certain types, staining these whilst leaving other structures unstained, or at most, stained so loosely that by correct washing the colour may be removed.

By a combination of two specific stains, or of a specific and a general stain, the whole of the specimen may be stained in various depths of one colour, whilst some particular constituent may be stained in a contrasting colour, thus rendering its presence more conspicuous, and its structure more easily followed.

Complete familiarity with a few stains is far better than a casual acquaintance with many.

Single Staining. From the many methods found in the literature, three have been selected as illustrating the chief types of process.

1. The specimen is placed in a drop of the stain on a slide, left immersed for a minute or so, and then covered with a cover glass for examination. This method is chiefly of value when examining a powder or section roughly in a general qualitative analysis.

2. The specimen is placed in a little of the stain in a small watch glass, which is covered with a slightly larger watch glass to prevent evaporation. The staining continues for some hours, after which the object is examined on the slide in a suitable medium.

3. The substance is placed in the stain, as in the two preceding methods, and after the correct length of time, is washed with some solvent or reagent which removes the stain from the elements which do not absorb the stain firmly. This washing process is extremely important, and it cannot be emphasized too strongly that the success of staining particularly with selective stains, depends more on the washing operation than on the staining proper. In some cases it is advisable to overstain deliberately, and to wash very thoroughly.

Double Staining. This does not differ in principle from single staining, and provided that the most firmly-fixed stain is applied first, there is no essential detail which need be varied when two staining methods are to be combined. The most important analytical stain is probably the haematoxylin-safranine mixture, but aniline blue: magenta, picro-carmine, and other combinations have their special uses.

HAEMATOXYLIN. The Delafield formula (No. 50) is very useful; although in recent years it has been to some extent replaced by aniline blue, it is still the best stain for cellulose walls and tissues, a fine purple colour being obtained. Sporogenous and similar tissues are also quite well stained. Starch, lignified and suberized tissues, and chromatophores are not stained, or only very lightly to a brown tint. It can be most strongly recommended as the best general cellulose stain available. It is tedious to prepare, and there is no advantage to be gained by the use of a "home-made" preparation. Unlignified and unsuberized membranes are stained a deep violet; other structures are unstained, or become yellowish-brown in colour.

It is most important that thorough washing should follow fixing. The preparation should be graduated into 40–50 per cent alcohol before staining. The section is then submerged in a large drop of the stain, and allowed to stand for from 10 to 30 min. When the staining has proceeded sufficiently far, the section is rinsed two or three times in 25 per cent alcohol (if necessary very slightly acid), and if not to be counterstained, is graduated to 90 per cent alcohol as a preliminary to mounting.

Counterstaining with safranine. This double-staining method is excellent for vascular tissue. The section is first washed with alcohol of the same strength as the safranine solution, or with water, if an aqueous solution is to be employed. It is then immersed in the safranine, and allowed to stand for 5 min., covered with a clock glass to minimize evaporation. The stain is drained off, the specimen washed with 50 per cent alcohol until no further red is extracted, and graduated to 90 per cent alcohol.

If the section must be examined immediately, the specimen, washed clean with 50 per cent alcohol, may be dehydrated on the slide by adding successive drops of 100 per cent alcohol, never allowing it to become dry. A drop of xylol is then placed in a clean hollow slide and the section transferred to it. When the preparation is cleared, the xylol is removed by filter paper, the section is picked up with a needle, and is placed on a drop of Canada balsam. When it has sunk to the bottom the cover glass is placed on and the specimen examined.

A further method of treatment of a stained section is to transfer to thin Canada balsam, by the methods given later, and to leave in this until such time as it may be conveniently examined. The advantage of this is that the clearing agent is well removed, which, in the case of clove oil, prevents subsequent browning of the mount.

Counterstaining with eosine (or *erythrosine*) is also a good method.

SAFRANINE. A dilute aqueous solution is a general stain for colourless transparent tissues. For convenience in processing, an alcoholic solution may be used, of the same strength as the stain which is to be used in conjunction with it. Mixed with aniline water, it is employed as a selective stain for lignified and suberized tissue, the stain being fast to washing with acid alcohol. Staining takes from two hours to a day. Lignified structures (e.g. in vascular bundles) are stained bright red.

BORAX CARMINE (Grenacher). (No. 45.) The colour of this stain is practically permanent in glycerine jelly. It is only recently receiving the attention which it deserves, as a stain for developing tissue. Staining takes from a quarter to half an hour; washing should consist of a rinse in acid alcohol, followed by steeping in alcohol for an hour. This stain is general rather than specific.

Counterstaining with malachite green (for formed or lignified tissue). Stain for a quarter of an hour or more, rinse quickly in two or three changes of alcohol, and as soon as the carmine stands out distinctly, clear as usual.

ALKANNA TINCTURE. (No. 48.) Dilute the stock stain with an equal quantity of water, immediately before use. Staining is carried out cold for a few hours. Fixed oils and fats are stained red; certain resins and rubber-like substances also stain; lignified and suberized tissues stain to some extent, but not so deeply as do oils.

CORALLIN SODA. (No. 47.) Callus plates and sieve tubes are stained red; lignified tissue, starch, and mucilage, pink. It is a good plan to overstain, and wash out with 4 per cent sodium carbonate solution. The stain is not permanent. A fresh solution must be used.

ANILINE BLUE. Unlignified and unsuberized cellulose are selectively stained, fast to clove oil, alcohol and xylol.

Counterstaining with Magenta. The colorations given are—

Pale magenta: Pith, hairs.

Deep magenta: Cellular tissue, liber cells.

Pale blue: Cuticle, pitted vessels.

Deep blue: Cambium, lactiferous vessels, parenchyma, epidermis, spiral vessels of medullary sheath.

Counterstaining with Fuchsin. After staining, the lignified elements appear red against a blue ground of the unlignified structures.

METHYLENE BLUE. Mucilage and pectic matter are stained, fast to washing with alcohol or glycerine. Pure cellulose is not stained; lignified and suberized tissues are stained, but are easily decolorized by washing with alcohol or glycerine. It is not of first importance as a botanical stain, though often useful.

RUTHENIUM RED. (No. 44.) A freshly-prepared solution stains many mucilages a bright pink.

SUDAN-GLYCERINE. (No. 17.) Suberized walls, e.g. in secretion cells, and fixed and volatile oils, are stained on gentle warming.

BISMARK BROWN. A useful mucilage stain. Pectic matter is also stained, whilst cellulose walls are coloured, though not with the precision of haematoxylin. A filtered saturated aqueous solution is employed, or a 2 per cent solution in 70 per cent alcohol.

EOSINE. Aleurone grains are stained pink, and cellulose walls red. For this latter purpose, it may be used as a counter stain to Grams violet; a convenient method is to add it to the clearing agent. *Erythrosine* gives greater but similar differentiation.

PICRO CARMINE. (No. 46.) A double stain, useful for wood sections. After treatment with the mixed colouring matters, wash a few times with 50 per cent alcohol, soak in two changes of an alcoholic solution of ammonium picrate for an hour each, wash lightly in alcohol, and clear.

PICRIC ACID. (No. 29.) Animal fibres are stained yellow. Aleurone grains are also stained. The coloration produced on other elements is easily washed out with water.

BRAZELIN. (No. 49.) Cover the section with the mordant, and stand for an hour. Pour off the mordant, rinse in 75 per cent alcohol, stain for several hours, rinse several times with 90 per cent alcohol and clear.

PHLOROGLUCINOL (No. 36), OR ANILINE HYDROCHLORIDE (No. 35). Stain lignified membranes. The colour of the last mentioned is permanent, the other two are not, when mounted in glycerine jelly or Canada balsam. The section is steeped for 5 min., drained, and mounted in concentrated HCl. Lignified walls are stained red according to the amount of lignification.

BRAEMAR'S REAGENT. (No. 43.) Tannin gives a yellow precipitate, which is an excellent confirmatory test, because many plant constituents give a blue coloration with iron salts.

FERRIC CHLORIDE. (No. 39.) Tannin matters are stained blue. In place of the usual water solution, which is almost always acid, a solution of the perfectly dry salt in ether is to be recommended.

OSMIC ACID. (No. 28.) Unsaturated fatty bodies slowly become dark brown. Saturated bodies, such as stearic acid or palmitic acid glycerides, are unstained.

ALPHA NAPHTHOL. (No. 34.) Inuline produces a violet coloration, if the section is steeped for a minute in the naphthol, drained, and mounted in concentrated sulphuric acid. Gentle warming may be necessary.

BENZOPURPURINE stains cellulose and callus, but not pectic matter.

NIGROSINE. Stains suberized or lignified tissue but not pectic.

GENTIAN VIOLET. This important stain does not keep very well, washes out very easily with alcohol, and is also dissolved by clove oil, which is often employed in differentiation. Lignified tissue is hardly stained, starch and cellulose stain deeply.

Bismark brown is a useful counterstain.

Examination Media. The factors governing the choice of the medium in which the specimen is to be mounted are chiefly the fineness of detail which it is necessary to observe; the type of detail, i.e. internal structure or surface markings; the condition of the substance, its bulk, thickness, water content, etc.; its refractive index; and the fixing or staining processes to which it has been subjected.

The medium itself may be simply air, an aqueous solution, a resinous body dissolved in a solvent, or an oil, amongst other possibilities.

The visibility of a structure under the microscope is due to a difference in refractive index between the substance and the surrounding medium. Theoretically, a body mounted in a medium of exactly the same refractive index as itself will be invisible, but in practice, this is never completely the case. There are invariably small amounts of surface impurities which indicate the boundaries, because they have a different refractive index from that of the medium. In addition, only a few objects are optically homogeneous, the refractive indices of the various components differing to some extent, with the result that internal structures are visible, even when the outside surface is almost invisible, and, indeed, this condition is the best for the observation of internal constitution.

Further, as explained in the section dealing with the calculation of refractive index, the relative n for various parts of the spectrum is different for solids and liquids. The extreme ends of the spectrum are therefore refracted to different degrees by substance and mountant, even when the refractive indices are exactly matched for the part of the spectrum mostly employed in visual work, that is, the yellow-green. In consequence, coloured fringes are seen round the boundaries of the object.

When the refractive indices of object and mountant differ, "refraction shadows" are produced, which define the structure. This is required for the observation of the surface or outline of a substance, but the difference in n should not be too great or the refraction shadows become too coarse and thick, thus preventing fine detail from being clearly discerned.

According as the n of the medium is greater or less than that of the object, the refraction shadow will be thrown outside or inside the specimen. In general, it is better for the medium to have the slightly lower n. For use with very high powers, and more particularly with immersion objectives, the refractive index should be sufficiently high to allow full use to be made of the aperture of both condensers and objective. The resolving power of an expensive wide aperture objective may be reduced considerably by the use of a medium of low refractive index.

A further and important property of a mounting medium is its penetrative power, which to some extent depends on its viscosity. A thick mass of tissue properly penetrated, becomes transparent, or "cleared," and the general arrangement of the structures or tissues is much more easily examined. The term "clearing," however, is often used to include the visual effect produced by other means than by the equalization of the refractive indices of substance and mountant, a simple example being the clearing action of water in dissolving out certain cell contents. This type of action has already been discussed.

It goes without saying, of course, that the examination medium must be colourless and transparent, and must, when permanent mounts are required, keep its transparency for many years, without any tendency to clouding or crystallization.

Examination media may be divided into several classes, according to their properties.

1. Non-aqueous media.
2. Aqueous media.
3. Air.

The first two may be further subdivided into—

(*a*) Examination media which by setting on cooling, or by evaporation of a solvent, leaving behind a solid substance, furnish permanent mounts.

(*b*) Those media which are suitable for temporary examination only, but are unsuitable for permanent mounts by reason of their volatility, or because they do not fix the cover glass firmly to the slide, or because they have some solvent or other detrimental action upon the preparation.

(*c*) Media of class (*b*) which, being otherwise suitable, may be converted into permanent mounts by sealing the edges of the cover glass with some cement, which prevents evaporation, and at the same time is sufficiently strong to fix the cover glass firmly to the slide. This operation is usually called "ringing."

1a. Permanent Non-aqueous Mountants.

CANADA BALSAM. (No. 77.) This resin, dissolved in xylol, is now almost universally used for permanent mounts. The solution must not be too dilute or shrinkage occurs on drying, leaving air bubbles round the edges of the cover glass; if very concentrated it is too viscous for efficient preparation. The slightest acidity will cause many stained preparations to fade.

Unfortunately, a lengthy process of manipulation is necessary in preparing a specimen for mounting in Canada balsam owing to the fact that it is completely immiscible with water and alcohol. This preparatory process for the treatment of a watery or damp substance comprises the following steps—

1. Transference to alcohol (dehydration).
2. Removal of alcohol by a substance miscible with both alcohol and Canada balsam (clearing).
3. Transference from the clearing agent to Canada balsam.

Various short cuts have been proposed, such as the use of aniline, to combine steps 1 and 2, but have not been widely adopted, except for rough work. Indeed, the tendency of the best workers seems to lie in the opposite direction, a further step being sometimes interposed between 1 and 2 by the use of a chloroform treatment, and occasionally between 2 and 3 (when using an oil), by the introduction of a xylol bath. These refinements are unnecessary in ordinary analytical work.

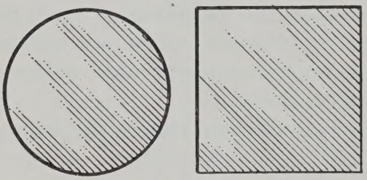

FIG. 121. COVER GLASSES

Dehydration. The usual dehydrating agent is alcohol. Delicate tissues, and cells containing much water, are strongly altered in shape if placed directly into concentrated alcohol; this is especially the case if the specimen is not fixed, or if an unsuitable agent has been employed.

It is the usual practice, therefore, gradually to increase the concentration of the alcohol employed, by transferring the specimen from dilute solutions progressively into stronger ones, an operation known as grading, or graduating. A suitable series of concentrations is 10, 30, 60, and 90 per cent alcohol, but more delicate structures, such as certain algae, require additional intermediate grades for the best results, to minimize plasmolysis. The section, or other material, is left in the earlier grades from one to two hours, the time increasing with the concentration to several hours. The specimen may be preserved in the final concentration of alcohol indefinitely before proceeding to the next step, though the last grade should be changed at least once, especially if the object requires preservation in absolute alcohol. Access of moisture should be guarded against very carefully when storing absolute alcohol.

Thick bodies require a long time in the alcohol, and it is better to err on the right side, both in the number of grades and in the time of soaking, than to discover later in the mounting that the preparation contains residues of water and is thus useless, or that the cell structure has become distorted by the too rapid withdrawal of water by the alcohol.

A second method which may be used is to soak the substance in 10 per cent glycerine, and to allow the glycerine to evaporate in the air to a constant weight. From this solution, transference may be effected into absolute alcohol without collapse.

Clearing. The removal of alcohol is usually termed clearing, though, as will be seen, clearing agents proper combine other functions than the replacement of alcohol, which will not mix with balsam, by a liquid which is miscible. Most clearing agents, it is true, may also be used as examination media, though they only become permanent mounts by ringing the cover glass with a varnish which will prevent evaporation, and will fix the cover glass so firmly that it can be wiped after examination with an immersion lens, or for cleaning. The so-called "clearing" before paraffin sectioning is really the replacement of alcohol by a paraffin solvent. The more important compounds are given below in section 1b.

A good clearing agent will possess the following properties—

1. Miscibility with both alcohol and the Canada balsam type of mountant.
2. No solvent action on aniline stains.
3. Slow rate of evaporation.
4. Sufficiently low viscosity as not to interfere with the penetration of the mounting medium, or of paraffin or collodion, if sections are to be cut after clearing.
5. Clearing action on alcoholic preparations.

The clearing is carried out as follows: More than enough alcohol than will cover the object is placed in a short but wide test tube, and by means of a pipette an equal volume of clearing agent is run in as a layer beneath the alcohol. The object is lowered on to the surface of the alcohol, and immediately sinks down to the interface. After some time it begins to sink slowly through the clearing agent; when it reaches the bottom of the tube, and no refraction lines can be observed in the clearing agent due to the alcohol, the preparation may be considered cleared.

GUM DAMMAR IN BENZINE. This may be used to replace Canada balsam. Its refractive index is slightly lower.

PIPERINE ($n = 1{\cdot}64$) is used in place of Canada balsam when a higher refractive index is required. The pure substance should be fused and kept at 180° C. for an hour, then slowly cooled. After this treatment it will not crystallise for many years, but it is not to be recommended for mounts of extreme permanency.

EUPARAL. This is a synthetic resin mountant which has a n of 1·48 but hardens to 1·53. It is less coloured than Canada balsam.

HYRAX. This is also a synthetic resin, n 1·63, hardening to 1·75 +, used when a high n is required.

1b. Temporary Non-aqueous mounts. ALCOHOL ($n = 1{\cdot}37$). Alcohol is so volatile that its use is attended with many disadvantages; a preparation must be observed immediately. It is, however, often employed for examination since so many objects are preserved in alcohol for reference, but its use should be confined to searching out a portion which is to be mounted more permanently. It can be used for watery substances.

XYLENE ($n = 1{\cdot}49$) is less volatile than alcohol, and its refractive index is quite good. It can easily be removed by evaporation, and for the preliminary examination of perfectly dry powders or other preparations which may later require to be permanently mounted in Canada balsam, it can be recommended. It is rather too volatile for use as an examination medium in general work. Objects cannot, however, be transferred from either water or glycerine, and only with difficulty from alcohol, directly into xylol, and in these cases, therefore, the usual process for mounting in Canada balsam must be employed. Xylol will, however, clear from absolute alcohol, though the modern custom is to use a series of absolute alcohol: xylol mixtures, much in the same way as various strengths of alcohol, are used for dehydration. The tendency to-day is definitely towards the use of xylol as a clearing agent (instead of the older oils), subsequent to the perfect dehydration of the specimen. Its small tolerance for water makes work even in a damp atmosphere somewhat difficult, but it is, on the whole, the best clearing agent at present available, especially for mounting in balsam and for paraffin sectioning.

BROMOFORM ($n = 1{\cdot}57$). The high refractive index makes this compound useful in some cases, and it has the further advantage of being miscible with both alcohol and Canada balsam.

ANILINE ($n = 1{\cdot}59$). Pure dry aniline (dehydrated with KOH) will take up 4 per cent of water, and provided a considerable excess of aniline be employed, and the watery body be steeped in it for some time, watery substances may be transferred directly into aniline. This reagent will, of course, clear from 75 per cent alcohol, or from pure alcohol, in addition to clearing from water. It has certain advantages in the treatment of sections cut by the celluloid process, and it may be used as a clearing agent for sections to be cut by the paraffin method. After treatment with aniline the preparation may be transferred directly into xylol, and from thence into

Canada balsam, though the longer and older method is to be preferred.

CEDAR-WOOD OIL ($n = 1{\cdot}515$). For general use, and particularly for delicate tissues, this oil is excellent. It clears rather like C.B., and will clear from 95 per cent alcohol without distortion. Aniline stains are not dissolved, and it penetrates well. In contrast to clove oil, it does not oxidize to a dark colour. It is suitable as a preliminary clearing agent for paraffin section cutting. Good cedar-wood clearing oil is difficult to obtain, except from reputed microscopic reagent dealers.

CLOVE OIL ($n = 1{\cdot}53$). One of the oldest of clearing agents, it has still a wide field of utility, in spite of its tendency to go brown with age, and to make material brittle. It clears more than balsam; absolute alcohol is preferable to 95 per cent for dehydration. It washes out many stains, and is now often replaced by xylol, which, however, mixes less readily with alcohol. Quite often the clove oil clearing is followed by a xylol bath, in order to prevent the fading which often gradually occurs with clove oil; when stains are used which are unaffected by clove oil, the precaution is unnecessary. Clove oil cannot be used to pre-clear for paraffin sections; and it dissolves collodion.

PHENOL. A concentrated solution in alcohol is an excellent clearing agent for watery preparations, but must be used for examination only, as it is not suitable as an intermediate stage for mounting in balsam. It should also not be used for the softer structures, as these shrink afterwards on treatment with a true clearing agent preparatory to mounting in balsam. Its chief value lies in the fact that preparations in water may be cleared directly.

A clearing agent with phenol as a base is due to *Eycleshymer*. It is a mixture of equal parts of cedar-wood oil, bergamot oil, and phenol, which clears from 95 per cent alcohol and does not dissolve collodion.

LIQUID PARAFFIN. According to Coles (*Lancet*, April, 1911), this liquid (when ringed, of course) keeps the colour of preparations stained with aniline dyes better even than Grübler's neutral Canada balsam in xylol.

1c. Ringing Non-aqueous Media. Ring with Canada balsam then with gold size etc., as described on page 197.

2a. Permanent Aqueous Mountants. GLYCERINE JELLY (n=1.47). (No. 76.) This most useful mountant may be recommended for almost dry bodies, such as textile fibres, and for all watery preparations; correctly employed, it furnishes mounts which are permanent for all practical purposes. The author has mounts in this medium which are considerably over twenty-five years old, and are still excellent. Its chief disadvantages are that it exerts a certain swelling action, and slowly dissolves substances which are soluble in water. It also dissolves calcium carbonate very slowly, and the phenol content seems to have a clearing action on starchy structures after some months. Its refractive index is somewhat low, but, nevertheless, high enough for all but the most exacting work.

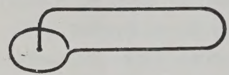

Fig. 122. Wire Clip for Holding a Cover Glass in Position Until the Glycerine Jelly has Set

A drop of the jelly, which has been melted in warm water, is placed on the slide, and the object is placed on the top of the solidified drop. The slide is held over a small Bunsen flame until the jelly melts, which is indicated by an increase in the diameter of the base of the drop, and is immediately withdrawn from the flame. The drop should never be allowed to boil, as this introduces a bubble and damages the object. A cover glass is now lowered gently on to the drop, and its weight causes the specimen to sink through the jelly. The cover circle is pressed down gently until the drop has set solid, and the mount is held under the cold-water tap for a few seconds to make the jelly firm. The excess of mountant which has been squeezed out round the cover glass may be wiped away with a rag, or scraped off with a penknife. Air bubbles which may have been introduced, can be removed by boiling the mount for a second or so, holding on the cover glass with a wire clip. Of course, many specimens would be damaged by this treatment.

2b. Temporary Aqueous Mountant. WATER ($n = 1{\cdot}33$). Its low index of refraction renders it unsuitable for examination under high powers, and it also has the disadvantage that it exerts a pronounced swelling action on many tissues, or affects the size and shape of cells by osmotic action. The addition of from 0·5 per cent to 1·0 per cent of NaCl reduces this osmotic action to some extent. It also has a strong solvent action which in many instances is fatal to successful observation. Its chief use is in the rough examination of textile fibres; mineral powders, etc., which are undissolved by it; in inorganic microanalysis; and occasionally in the examination of specimens which have been fixed with osmic acid or heavy metal salts.

GIBSON'S MEDIUM. (No. 111.) This may be recommended for the observation of fine cellular detail in plant structure.

STEPHENSON'S MEDIUM ($n = 1.68$. S.G. = 3.02.) (No. 112.) This is an excellent medium for quick observation of cell structures which contain water, or preparations which are not dry, but which yet require to be examined in a medium of high refractive index. It may, be diluted down to about $n = 1.55$ for ordinary work with advantage and can then take the place of Canada balsam. Its use avoids the long process which must be employed to transfer a wet body into Canada balsam. It exerts an appreciable osmotic action, but does not cause swelling. Being a mercuric compound, it also exerts a fixative action. It is not suitable for permanent mounts.

GLYCERINE. Pure glycerine has an excellent clearing action, but is awkward to use because it is so decidedly hygroscopic. The transference of the more delicate cell structures directly into glycerine causes a pronounced shrinkage, due to the hygroscopic nature of the mountant, and, therefore, the glycerine must gradually be increased in strength. This may be done by placing the substance in a dilute solution of glycerine, and leaving it in a desiccator for some days, until sufficiently concentrated. A mount in concentrated glycerine must be kept in a desiccator, or within a few hours the mount will have absorbed so much moisture from the air that the slide will be flooded. A more permanent mount may be obtained by painting the edge with melted glycerine jelly. Pure glycerine dissolves calcium carbonate.

LIQUID PHENOL. Phenol, to which just enough water has been added to make it liquid at room temperatures, is an excellent clearing agent for certain types of substance; it will even clear from water. Thus, it renders starch almost invisible (though the reaction with polarized light is still obtained) and, in consequence, a powder, such as self-raising flour, mounted in the medium, shows quite plainly any inorganic additions, rendering it unnecessary to separate the constituent substances by treatment with an acid, malt extract, or other agent, which will remove the starch. Phenol should not be employed for delicate plant structures, as shrinkage occurs during later treatment.

CHLORAL HYDRATE. A mixture of five parts with two of water is commonly employed. It dissolves cell contents such as proteins and starches, the latter, however, requiring the application of heat.

2c. Ringing Aqueous Mounts. Water mounts should first be ringed with thin glycerine jelly, and after twenty-four hours again ringed with a thin line of Canada balsam, in order to seal them. The next step is to ring with gold size, which is best done by spinning the ringing table, and painting a ring of the gold size rather larger than the mount, gradually working in until the edge of the cover circle is properly painted. This first layer should be thin, and should be allowed to stand for a day or two to harden, after which a thicker ring may be put on. If the ring is too thin, it will easily break, but too thick a ring will interfere with observation under high-power objectives with a short working distance.

Glycerine jelly mounts should be sealed with Canada balsam, and then treated as above.

3. Mounting in Air. There are few uses for a mount in air, its chief value being for examination under very low powers, or for the observation of almost perfectly flat structures, such as metallurgical sections. Occasionally, however, as in Herzog's method of section cutting of artificial silks, the air method is the only one which can be employed.

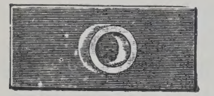

FIG. 123. RING CELL FOR MOUNTING IN AIR

A ring of some kind is necessary, which, in the case of thin objects, may be cut out of thin cardboard, e.g. a postcard, but in the case of thicker objects should be a glass or metal ring. This is affixed to the slide as follows: a tacky cement ring is (No. 84) painted on the perfectly clean slide, and allowed to dry. This is then coated with a further layer, on which the ring is pressed firmly, and left to set. The specimen is placed inside, being fixed in position in a suitable mountant, e.g. tannin glycerine (No. 80.) The top surface of the ring is now painted with the cement, and a cover glass placed in position. The edges may be sealed with Canada balsam, and finally, after some days, ringed with old gold size. (No. 86.)

Mounting Dry Powders. There are two methods which give good results. The more convenient is to place a little of the powder on a cover glass; it may be distributed evenly over the surface by tapping the underneath side with a pencil. The cover glass is then turned over, and gently lowered, with the powder adhering to the underside, on to a drop of the medium on the slide.

When this method is unsuitable, either because the powder will not adhere to the cover circle, or because larger particles tend to be lost, a little of the powder may be dusted on to a drop of the medium on the slide by tapping the underside of a cover glass on which the powder has been placed. In this case the powder should be allowed to remain for a few minutes before finally placing a clean cover glass in position.

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See also page 220.

CHAPTER X

BOTANICAL STRUCTURES

In the following pages a scheme is presented for the systematic examination of a vegetable substance. A knowledge is presumed of botanical structure, but readers who are unfamiliar with the subject may be recommended to read *Plant Anatomy*, by W. C. Stevens, and pages 40-71 in *Strasburger's Textbook of Botany* (1921 Edition).

TABLE III

General Scheme for Identification

<table>
<tr><td>Chlorophyll Present</td><td colspan="4">Chlorophyll Absent</td></tr>
<tr><td rowspan="5">Leaves
Leaf stalks
Stem
Calyx</td><td>Vessels Present</td><td colspan="3">Vessels Absent</td></tr>
<tr><td rowspan="4">Wood
Roots
Rhyzomes</td><td>Sieve Tubes Present</td><td colspan="2">Sieve Tubes Absent</td></tr>
<tr><td rowspan="3">Cork
Barks</td><td>Aleurone Grains Present</td><td>Aleurone Grains Absent</td></tr>
<tr><td rowspan="2">Seeds
Fruits</td><td>Pollen Present</td></tr>
<tr><td>Flowers</td></tr>
</table>

Chlorophyll. Chlorophyll is found in the leaves, leaf stalks, petioles, stems, and calyx. Under certain circumstances it changes to a brown derivative.

Leaves. If the substance is in a powdered form, it should be remembered that large thick-walled vessels, and much lignified tissue should be absent, whilst it is only very rarely that cork cells, aleurone grains, and reserve materials, such as fat, or starch, are present.

The epidermis is usually a single layer of cells; the shape of the cells varies with the different species, but may be taken as fairly constant within any one species. These cells usually appear rectangular in the cross-section.

The cuticle on evergreen trees and shrubs is, as a rule, much thicker than that on herbaceous plants. It is usually quite smooth, though ridges and protuberances are to be found in certain plants.

Stomata are present on almost all leaves, and are usually evenly distributed all over the surface. Any special groupings should therefore be noticed. The cells round the stomata have often some special arrangement which may be characteristic.

Hairs are found very generally. They are usually present on stems and veins or guarding stomata. They vary enormously in length, shape, and structure, and are most important for identification purposes, since they are constant in type in any one species.

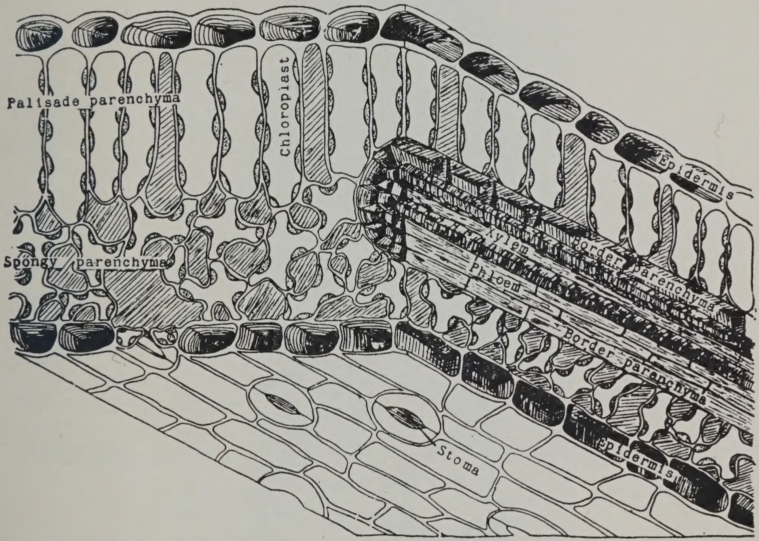

FIG. 124. A TYPICAL LEAF IN THE REGION OF ONE OF THE LATERAL VEINS

The shaded parts amongst the palisade and spongy parenchyma represent intercellular spaces

(After Stevens)

Unicellular hairs are composed of a single cell.

Pluricellular hairs are composed of many cells.

Uniserial hairs have the cells arranged in one row only.

Pluriserial hairs have the cells distributed in several rows. In many cases these hairs carry at the top a special type of cell which secretes oil or resin. This cell, which is then known as a gland, may be unicellular, but is often split up into two, four, or eight compartments by means of walls, which are usually vertical; sometimes horizontal; occasionally both kinds of walls are present.

The mesophyll (the region between the upper and lower epidermis) in most cases may be considered to consist of two sections, the layer next to the epidermis, known as the palisade, having cells differing in shape from the parenchyma or spongy tissue of the main body of the mesophyll. The structure is then termed heterogeneous, whilst if the parenchyma be absent, it is termed homogeneous mesophyll. This latter form is not very common, and hence its occurrence is valuable in identification. Again, the elements in the mesophyll are usually dorsiventral, and any isobilateral cells should also be noted specially. Apart from these points, the mesophyll

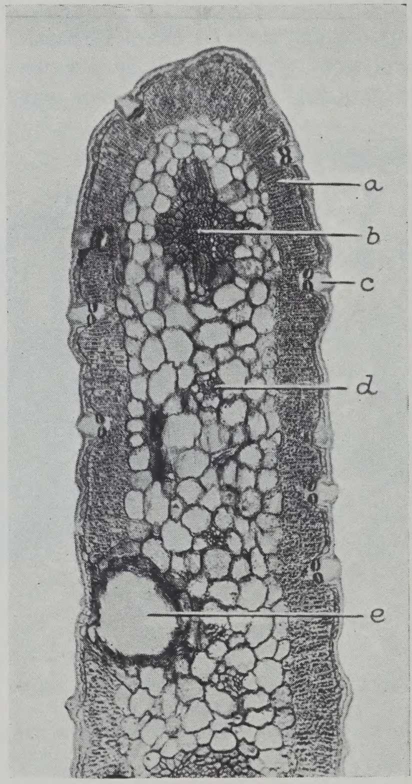

FIG. 125. TRANSVERSE SECTION OF THE LEAF OF "METROSIDEROS VERNI"

A = Palisade layer.
B = Primary vascular bundle.
C = Stoma.
D = Secondary vascular bundle.
E = Oil gland.

layer is chiefly important from the fact that several special kinds of cells are present in certain species only.

Midrib and lateral veins usually show a very similar structure to the stem, and have few special features. Beneath the epidermis is a layer of more or less collenchymatous elongated cells which have thickened walls, and which gradually merge into the usual parenchymatous cells. These are thin walled, and are elongated in shrubs, but tend to be rounded in herbaceous plants. Occasionally pericyclic fibres are present in the bast and wood, and these may be characteristic.

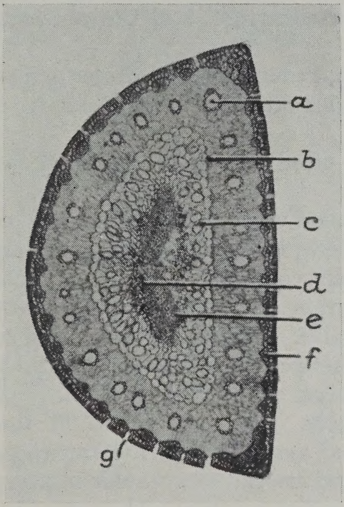

Fig. 126. Transverse Section of Leaf of "Pinus Sylvestris" Common Pine

A = Resin duct.
B = Endodermis.
C = Transfusion tissue.
D = Phloem.
E = Xylem (wood).
F = Epidermis.
G = Stomata.

Stem. The structure of the stem is in a general way analogous to that of the leaf.

The epidermis is a single row of cells, which may differ in shape largely. Stomata and hairs may be present, as in the leaf, and a coating of cuticle is usually to be seen.

The *Cortex*. Here, again, the cells adjacent to the epidermis tend to become collenchymatous, the main body being composed of parenchymatous cells, which are very much alike in all plants. The cortex may contain cells of a special type, such as palisade, sclerenchymatous cells, or fibres, lactiferous cells or vessels, and oil glands or cells. The parenchyma themselves may have special or unusual cell contents, such as calcium oxalate or carbonate, starch, tannin or oil, which all aid in identification.

The endodermis is the inner lining of the cortex, as the epidermis is the outer lining, but it is often difficult to trace on the older stems. The cells in appearance are thin-walled and rectangular; the walls may be lignified or suberized, and the cells may contain reserve materials.

The pericycle layer may consist of one or more rows of parenchymatous cells which often have changed more or less into sclerenchymatous fibres.

The bast ring is often very small and is of little importance for identification.

The wood is of little value in itself, but may contain interxylary bast, which, if present, is a useful piece of evidence.

The pith is usually composed of large parenchymatous cells, the walls of which are thin and often lignified. Interxylary bast may be present occasionally. Lactiferous vessels and cells are often found in certain species between the endodermis and the wood, and are highly important as an aid to identification.

Lactiferous Vessels. There are found in the bast of certain stems some unusual vessels which are rather conspicuous. They are large in size, and are full of granular matter which stains deep yellow with dilute iodine solution. They may be seen most readily in radial section, and in a suitable section are evidently not separated from each other, but are connected (anastomose) in a canal system.

They may be isolated for examination, by digesting on the water bath with 2 per cent KOH for the greater part of an hour, until by trial, the cortex is found to separate easily. Remove all tissue external to the wood, and after washing well, mount in dilute iodine.

These vessels occur principally in the following orders only: Papaveraceae, Compositae, Campanulaceae, Convolvulaceae, Olacineae, Euphorbinaceae. (Solerederer, anatomie der dicotolydonen.)

Lactiferous Cells. These also occur in the bast ring of stems, and are very similar to the lactiferous vessels in reactions and cell contents. In transverse section these cells seem much larger than the normal cells of the bast. They may be isolated by the same means as the lactiferous vessels, and are then seen to be long tubes showing side branches, which, however, do not anastomose with each other. They occur chiefly in the stems and leaves of the orders apocynaceae, asclepiadaceae, euphorbiaceae, urtucaceae.

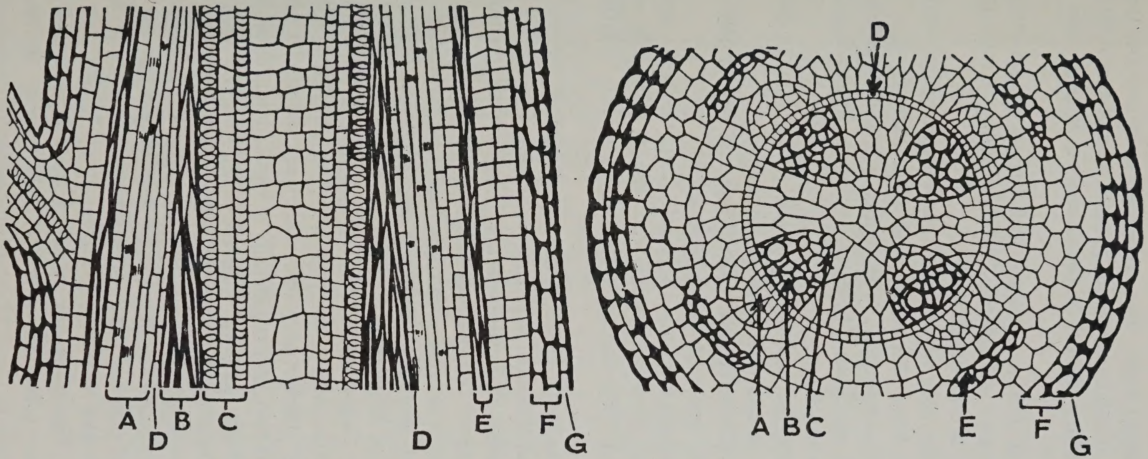

FIG. 127. TRANSVERSE AND LONGITUDINAL SECTION OF A TYPICAL STEM AT THE BEGINNING OF CAMBIAL ACTIVITY

(After Stevens)

A = Phloem from the procambium and cambium.
B = Xylem from the cambium.
C = Xylem from the procambium.
D = Cambium.
E = Bast fibres.
F = Collenshyma.
G = Epidermis.

CALYX. This is usually of the same structure as a leaf, and should be examined in the same way. The epidermis is important from the point of view of the stomata, and the presence or absence of any oil cells and calcium oxalate should be determined. It is of rare industrial occurrence.

Figs. 127, 128, and 129 are general indications of plant lay-out, and do not represent any one species. The following characteristics of the tissues shown are of importance.

COLLENCHYMA. The cell angles, and sometimes radial walls, are thickened. Old cells have the entire wall thickened.

PARENCHYMA (Prosenchyma). Cell walls are unthickened. Distributed throughout the plant, being the active, living foundation tissue, containing protoplasm.

STONE CELLS. These are not often present, but occur, for example, in pear fruit. Their function is to give strength.

STARCH SHEATH (Endodermis). A thin layer, often difficult to observe in stems, but more easily seen in roots.

BAST FIBRES (Sclerenchyma). Strengthening tissue, occurring in suitable positions, varying with the plant. In the square-stemmed dead nettle, for example, the bast occurs at the four angles.

PHLOEM. (Sieve tubes.) Until recently regarded as the main channels for the conduction of sap and sugar solutions. Sieve tubes always have a smaller "companion cell" containing protoplasm, which probably controls the phloem cell activity.

CAMBIUM. The cells of this layer are differentiated inwards to form the wood, and outwards to form the phloem. The layer is living and very active in growth.

XYLEM. (Wood.) Composed of xylem vessels, xylem tracheids, and parenchyma. The inner- or proto-xylem exhibits cells with spirally or annularly thickened walls, which are normally pitted.

Vessels present. When chlorophyll is absent, search should be made for vessels, which may be derived from wood, roots, or rhizomes. The characteristic features of these classes are given below, but it should be remembered that parenchymatous tissue filled with reserve material will point to a rhizome or root, whilst any medullary rays which consist of parenchyma often containing reserve material at right angles to the axis will indicate wood.

WOOD (XYLEM). The elements of wood are all produced by the cambium on its inner side. Though the cells now to be described are sufficiently definite in type, they often merge into each other by insensible degrees, and in such cases no hard and fast line of division may be drawn between them.

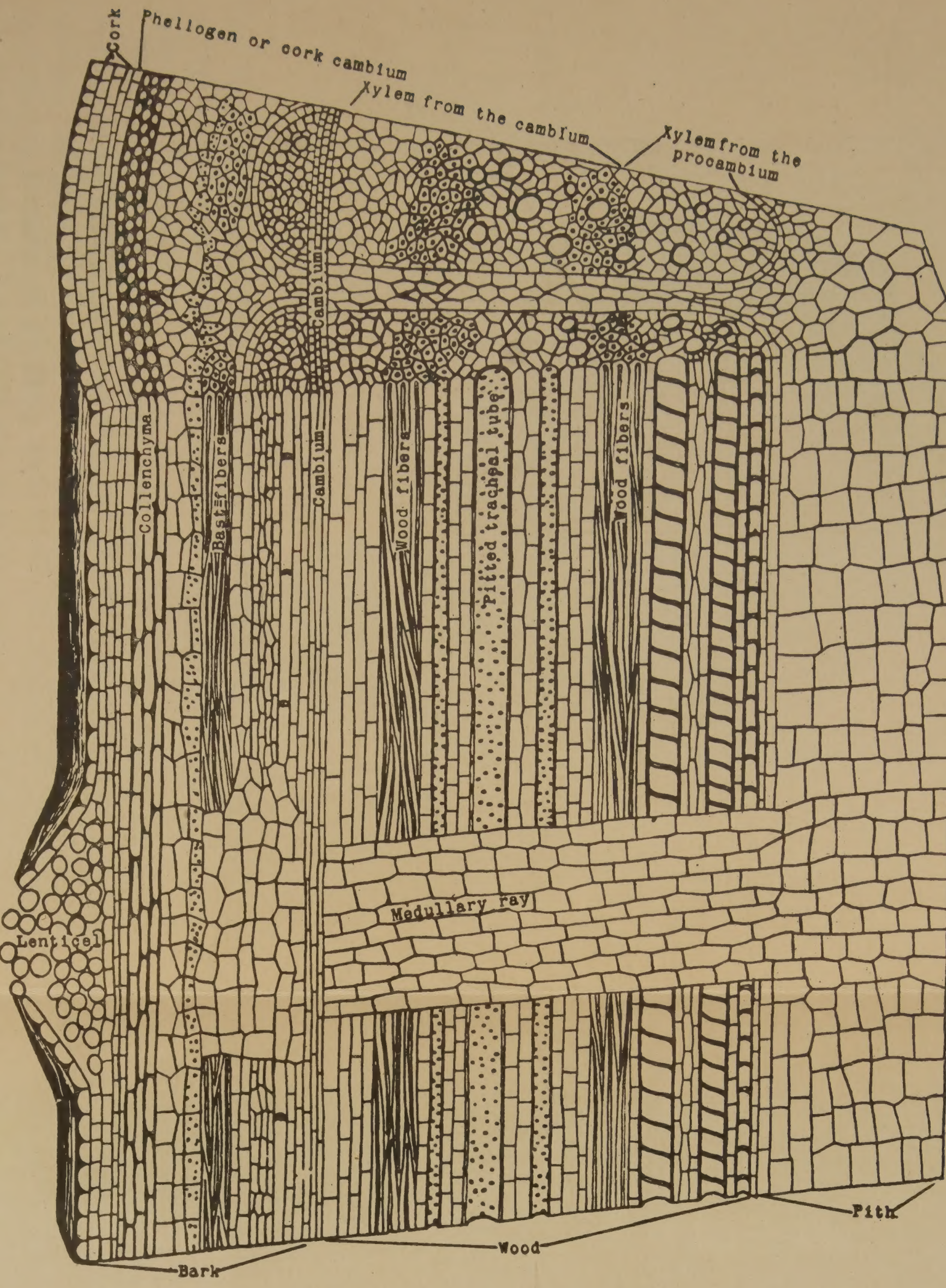
Cork
Phellogen or cork cambium
Xylem from the cambium
Xylem from the procambium
Collenchyma
Bast fibers
Cambium
Cambium
Wood fibers
Pitted tracheal tube
Wood fibers
Medullary ray
Lenticel
Bark
Wood
Pith

FIG. 128. DIAGRAM SHOWING THE TOPOGRAPHY AND CHARACTER OF THE TISSUES EVOLVED FROM THE PRIMARY MERISTEMS BEFORE THE BEGINNING OF CAMBIAL ACTIVITY
(*Stevens*)

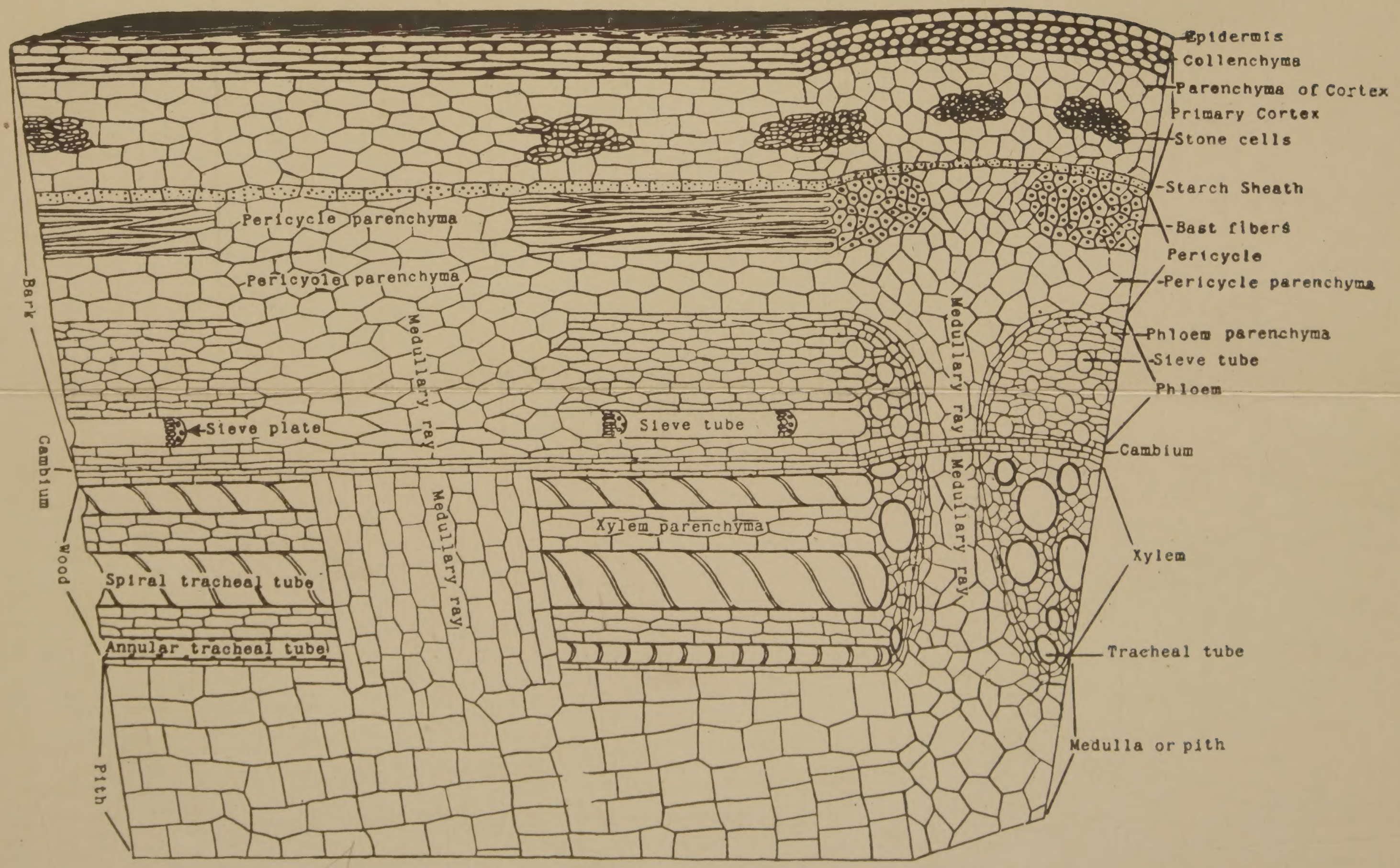
Epidermis
Collenchyma
Parenchyma of Cortex
Primary Cortex
Stone cells
Starch Sheath
Bast fibers
Pericycle
Pericycle parenchyma
Phloem parenchyma
Sieve tube
Phloem
Cambium
Xylem
Tracheal tube
Medulla or pith
Pericycle parenchyma
Pericycle parenchyma
Medullary ray
Medullary ray
Medullary ray
Medullary ray
Sieve plate
Sieve tube
Xylem parenchyma
Spiral tracheal tube
Annular tracheal tube
Bark
Cambium
Wood
Pith

FIG. 129. DIAGRAM SHOWING ADDITIONS TO THE PRIMARY TISSUES THROUGH THE ACTIVITY OF THE CAMBIUM AND PHELLOGEN
A later stage of Fig. 128 (*Stevens*)

These xylem vessels may be considered as a line or row of cambial cells which have in effect become one large cell owing to the adjacent end cell walls having become broken down or perforated. Hence, the length may vary considerably, and the width becomes the more important factor. Pits and other markings are found on the walls, and their shape and size are often characteristic. In the preparation of crude fibre, vessels are often broken up into the original individual cells.

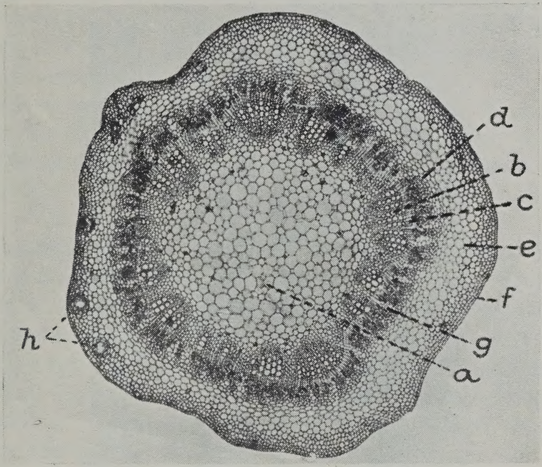

FIG. 130. TRANSVERSE SECTION OF STEM OF "GOSSYPIUM SP." COTTON PLANT

A = Pith.
B = Xylem.
C = Cambium ring.
D = Phloem.
E = Cortex.
F = Cuticle.
G = Medullary rays.
H = Resin cells.

Tracheids, though very similar in appearance to vessels, may be distinguished by the fact that they developed from one cambial cell, and are thus not formed by the partial fusion of several. In consequence, the remains of dividing walls which are displayed by vessels are usually absent. Small pits are, however, normally present, and in the woods of conifers may be quite large. Tracheids have normally no cell contents, since their chief use is the transmission of water. The forms which merge into wood parenchyma may, however, store some reserve material. True tracheids are smaller than vessels.

Fibrous cells (Xylem fibre) are the intermediate or transitional cells between tracheids and parenchyma. They are usually shorter and have more rounded ends than the latter. They may be thin-

or thick-walled, and may serve as storage cells, but their chief difference is that only one is produced from one cambial cell.

A single cambial cell may develop several wood parenchyma cells, and, in consequence, the end cell of the string is often very conical.

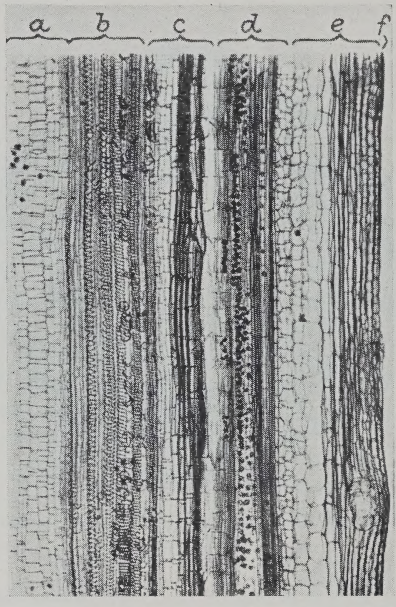

Fig. 131. Transverse Longitudinal Section of "Gossypium Sp." Cotton Plant

a = Pith.
b = Spiral vessels.
c = Cambium.
d = Phloem, containing crystal clusters
e = Cambium.
f = Cuticle.

The walls are often fairly thick and exhibit numerous pits. They often contain various cell contents.

The great bulk of wood is composed of wood fibres. They are of no importance to the wood except mechanically, and therefore reserve materials other than perhaps resin and oil are absent. They are long narrow blades, which taper at each end to a point. The walls do not show a great many pits, those present being mere narrow slits, often spirally arranged.

Medullary rays are structurally the same as the wood parenchyma, but are found in the wood at right angles to them, i.e. along the radius of the wood.

Roots and Rhizomes. Structurally these are not so complex as aerial stems, and hence the details are of great importance in identification. Many roots also pass gradually into rhizomes, and the general structures show few important differences.

In very young roots the bast and wood bundles are placed characteristically, but often this is not obvious in older roots. Rhizomes contain a pith which may be readily seen, but it must be remembered that some woods contain a parenchymatous tissue which very closely resembles this. Rhizomes in transverse section often show leaf traces which pass through into the stele, whilst roots never exhibit this structure.

The stele is separated by an endodermis from the cortex. The epidermis is not often to be seen, having been shed by the plant. The tegumentary layer is chiefly cork, and the cortex is often considerable. The wood bundles may be in a continuous or a diffuse ring.

The majority of medicinal rhizomes are from monocotyledons, which have closed bundles exhibiting no cambium. The bundle is often semi-surrounded by a crescent of sclerenchymatous fibres. Most medicinal roots, on the other hand, are from dicotyledons.

Both roots and rhizomes function largely as storage organs (though true roots act largely by absorbing water), hence the cell contents are very important. The reserve material is stored chiefly in the parenchymatous tissue, which is very considerable in extent. The size, shape, colour, thickness of walls, and nature of the pores of the parenchymatous cells, vessels, and fibres should also be noted.

Sieve tubes present. If vessels and chlorophyll are both absent, a preparation should be stained with corallin soda for sieve tubes. The presence of sieve tubes indicates bark. If bast fibres and sclerenchymatous cells are also present, a bark is indicated.

BARKS. Just as wood is considered to be that section of the stem which arises from the internal surface of the cambium, so barks may be defined for the present purpose as all structures which are produced outside the cambium, whether they are directly derived from the cambium, or otherwise.

It should be noted that the bark of certain roots and rhizomes contains all the elements mentioned below, with the exception, as a rule, of sclerenchymatous cells and fibres. It may also be pointed out that although a pure bark powder should contain no vessels, these may be present in small quantity from the wood, which is always found attached to some pieces of bark, and hasty conclusions should not be reached concerning adulteration.

CORK. This is developed from the phellogen, and is usually quite regular in structure, forming several layers or rings of cells which are also arranged in regular radial rows, like a brick wall. The amount produced varies greatly in different plants. Cork tissue, though usually present in powdered bark is, in certain cases, removed during the process of manufacture.

Individual cells have thin, yellow-brown, suberized walls, which are often lignified. The cell contents are usually some amorphous brownish matter akin to tannin. The cork tissue is very resistant to powdering, and in powdered drugs, for example, occurs as flattened fragments.

The phelloderm, or secondary cork, is also derived from the phellogen, but from the interior side. The arrangement is very similar to that of cork, in most cases, but the walls are neither suberized nor coloured. In old plants, the regular structure may be destroyed, and gradually the formation will merge into that of the primary cortex. Special features are rarely present.

The cortex, which is usually composed of parenchymatous cells, shows an outer layer near the phelloderm layer, which is composed of cells inclined to become irregularly collenchymatous. The cortical parenchymatous cells are usually broader and shorter than the bast parenchyma, and also, unlike these, exhibit quite pronounced spaces between the cells. Characteristic features are rare, the chief value of the layer being due to the presence of other elements such as glands; sclerenchymatous cells, which in many cases occur in patches; and the cell contents, which may comprise starch, calcium oxalate, oil, and (near the phelloderm) chlorophyll.

The bast ring layer constitutes the greater portion of the average bark, and the most characteristic elements are to be found therein.

The medullary rays of the wood usually continue through the cambium into this tissue, but it should be noted that the cell walls are thin and unlignified. The main bulk of the bast tissue, however, is composed of sieve tubes and bast parenchyma. These are rather alike in appearance, and search should be made for the sieve plate, which is often coated with callus. The thickness of the walls and size of the cross-section are also useful. The sieve tube is the only element present in all bark powders, and its presence indicates bast tissue.

Though the sieve tubes are of the utmost importance in proving the presence of a bark, they offer little information as to which particular bark is in question, and this must be decided largely by other elements present. Any sclerenchymatous cells and fibres will often occur at the outer end of the medullary rays. These should be closely examined, noting the quantity, arrangement (isolated or in groups), and orientation of the groups, the thickness of the walls, and the size and shape of pits and striations. The bast fibres are almost always present, but the sclerenchymatous cells are fairly often absent. Whilst the thin-walled bast fibres are easily broken up by grinding processes during manufacture, the thicker-walled sclerenchymatous fibres are mostly found undamaged.

The cell contents are very often characteristic. Starch grains are small and not often of very definite shape. Colouring matter quite frequently occurs. Oil glands and cells are most important, and in a powdered bark in which their shape has been destroyed by grinding, they may be detected by treatment with Sudan Red, and other stains for suberized tissue.

Aleurone grains. When sieve tubes are also absent, a seed or a fruit is probable. The presence of aleurone grains definitely proves the presence of a seed.

Protein matter is stored in seeds as small grains or globules, just as starch is stored as grains, and the granules, which may be amorphous or crystalline, are often very constant in shape and size in any one kind of plant. They are thus of great importance in identification, especially when the seed coats or other characteristic structures have been removed or are absent. The grains are usually accompanied in the cell by "fixed oil" and a so-called ground substance. The latter appears as amorphous, minute granules, insoluble in alcohol, glycerine, or fixed oils, but readily soluble in $\frac{1}{4}$ per cent KOH, limewater, up to 10 per cent NaCl., up to 20 per cent $MgSO_4$ sodium phosphate, and very often in water only.

The aleurone grains themselves may be amorphous or crystalline. The crystalline forms are not true crystals, because the angles vary, and the crystals swell under suitable treatment. They dissolve in a similar manner to the ground substance, but much less readily, and are only soluble easily in KOH. They are stained yellow by picric acid, brown by iodine, and red by Millons' reagent.

The globoids (the amorphous form) differ somewhat in their reactions, being, for example, insoluble in water and dilute alkali, but soluble in dilute acids and in sodium phosphate. They are also unstained by iodine or picric acid, and are normally quite small.

To examine these bodies a section of the substance is first extracted with a mixture of ether and alcohol on the water bath, which removes oil. It should then be mounted in alcohol, and irrigated with iodine solution, when the aleurone grains should stand out plainly, both the crystalloid and globoid forms being clearly visible. By now irrigating with dilute caustic potash, the crystalloid is dissolved, the globoid alone remaining.

A further substance present frequently along with the aleurone grains is calcium oxalate. One cell may contain a large number of small grains, or a few large ones, or a mixture of the two types. The grains themselves exhibit a great diversity in form, and may be accompanied by any mixture of globoids or crystalloids, the whole being embedded in a varying quantity of ground substance. Very often the calcium oxalate is present as crystals, needles, or more often in small rosettes.

The essential parts of a seed are the kernel and seed coat. The seed coats vary enormously in structure from one species to another, and there may be from one to three present, in addition possibly to a caruncle. The seed coats contain the most characteristic elements of a seed. The following layers should be sought for, and are best found in a cross-section.

Mucilaginous Layer. This, if present, is usually on the outside, and consists, as its name implies, of a layer of cells which secrete mucilage. Mucilage, whether as a secretion or a cell content, is colourless, and is easily passed over unnoticed. It is insoluble in alcohol or glycerine, but the various kinds differ considerably in their behaviour towards water, some swelling, and others dissolving. Irrigation of the section with basic lead acetate causes the mucilage to become granular and pale yellow. Certain stains may be employed as a means of detecting mucilage, but there is no one reagent which will demonstrate the presence of all kinds of this substance, although dilute indian ink has been suggested, the particles of which are absorbed by the surrounding tissue, but not by the mucilage, leaving it white. Corallin soda, ruthenium red, and bismark brown are the more important of the stains which colour mucilage.

Sclerenchymatous Layer. One or two of the outer rows of the seed-coat cells often become transformed into more or less sclerenchymatous cells and fibres, which frequently assume very characteristic shapes or structures.

Other Layers. The colouring matter of a seed is often contained in a special layer of cells. Occasionally distinct layers are found containing oil, or tannin; or again, rows of cells of a very strongly modified form are sometimes present.

Cell Contents. Starch is extremely common, and is usually mixed with small aleurone grains. Seeds which have oil as their reserve material in place of starch, usually have the aleurone grains much larger, and almost as characteristic in size and shape as the starch grains.

Kernel. This consists of the embryo, possibly surrounded by either a perisperm or an endosperm, or both. Aleurone grains are present in ripe seeds only.

FRUITS, in contrast to seeds, exhibit the presence of an epidermis with more or less distorted stomata, in addition to the aleurone grains. There is also present the so-called aleurone layer, and a large amount of parenchymatous tissue.

Pollen grains. Having carried the analysis thus far, a search should then be made for pollen grains, which indicate a flower. The petals should be sought, which may be coloured, and may have a pappilose epidermis. The endothecium of the anthers also exhibits characteristic features, such as spirally-thickened walls.

REACTIONS OF STRUCTURAL ELEMENTS. The reactions of most value in analytical work are summarized below under their appropriate heading. Typical reactions may be explored amongst the following substances—

Cell walls—cotton-wool. Lignocellulose—pine wood, e.g. match stalks. Suberized tissue—cork. Callus—sieve tubes from Curcubita. Calcium oxalate—Cactus stem. Calcium carbonate—Ficus elastica leaf. Oils—castor oil seed. Carbohydrates—beetroot, dahlia tuber. Starches—potato, wheat flour. Tannin—oak, horse chestnut. Proteids—pea cotyledon.

Cell walls. CELLULOSE WALL. Concentrated sulphuric acid dissolves cellulose. Ammoniacal copper oxide also effects solution. Iodine-sulphuric acid cr zinc chloride-iodine give a blue-violet coloration. Haematoxylin does not stain deeply. Aniline blue stains fast to alcohol and xylol.

LIGNIFIED MEMBRANE. Ammoniacal copper oxide is not a solvent. Iodine-sulphuric acid or zinc chloride-iodine gives a yellow brown coloration. Aniline sulphate, phloroglucinol, and thymol stain the membrane. Fuchsin stains fast; counterstain with haemotoxylin or aniline blue. Safranine-aniline water stains fast to acid alcohol. NaOCl destroys lignin.

SUBERIZED MEMBRANE. Insoluble in ammoniacal copper oxide or concentrated sulphuric acid. Iodine-sulphuric acid, etc., stain yellow-brown. Concentrated caustic potash gives a yellow colour cold, deepening on warming. Alkannin stains to a medium red tone after some hours. Safranin aniline water stains fast to acid alcohol; counterstain haematoxylin or aniline blue. Schultz's maceration process converts it finally into oily drops, but the structure is very resistant. Cyanin stains deeply, but tannin must first be destroyed by NaOCl solution.

CALLUS (SIEVE TUBES). Insoluble in ammoniacal copper oxide. Dissolved by concentrated calcium chloride, cold concentrated sulphuric acid, and cold 1 per cent caustic soda. Corallin soda and aniline blue stain well. Zinc chloride-iodine gives brick red to red-brown. Pectic matter (intercellular substance of unlignified and unsuberized structures). Methylene blue, fuchsin, and bismark brown stain well (contrast cellulose) fast to alcohol and glycerin (contrast lignified suberized membranes). Benzopurpurin, nigrosine, and induline do not stain.

Cell contents. SILICA. The substance is heated with concentrated sulphuric in order to obtain a "silica skeleton." This may be carried out on the cover glass until the preparation is quite white, and the acid has evaporated.

CALCIUM OXALATE. Structures containing crystals of the salt can be cleared by means of boiling phenol or chloral hydrate. The salt is soluble in hydrochloric acid, though large crystals, especially if embedded in mucilage, are rather slow in dissolving. Sulphuric acid slowly converts the crystals into needles of calcium sulphate. Barium chloride leaves the crystals unchanged. Long prisms are found, e.g. in quillaya bark, which may be exposed very clearly by boiling the structure in caustic potash. Acetic acid does not dissolve the oxalate.

CALCIUM SULPHATE. Barium chloride causes the crystals to become covered with fine deposits of barium sulphate. Calcium phosphate is precipitated as sphaero crystals during the alcohol dehydration. The crystals show the starch cross under polarized light, give the molybdate reaction, and are converted into sulphate by sulphuric acid.

CALCIUM CARBONATE. This usually occurs as a cell-wall deposit. Concentrated hydrochloric acid liberates small bubbles of CO_2. Sulphuric acid forms calcium sulphate needles. Acetic acid dissolves the carbonate.

IRON. This sometimes occurs. The usual test is by means of potassium ferrocyanide. Sections (cut by a steel knife) sometimes give a reaction for iron.

OILS. Soluble in the usual fat solvents. Alcannin stains the globules deep red after from 2 to ten hours. A mixture of concentrated KOH and ammonia (Molisch) saponifies them in about half an hour, forming soap in small needle crystals. Osmic acid stains unsaturated oil deep brown or black, but not saturated fats; tannin should first be extracted by boiling water, and the colour may be controlled by means of hydrogen peroxide. Cyanine stains in half an hour; over-staining is corrected by glycerine (lignified and suberized membranes are also stained).

CARBOHYDRATES. *Molisch's Test.* Cover the section with alpha naphthol solution, and add 1 to 3 drops of concentrated sulphuric acid. A violet coloration after 2 min. indicates cane sugar, milk sugar, glucose, laevulose, maltose, or inulin. No coloration is caused by inosite or mannite. Insoluble carbohydrates react only after half an hour or more, and then only consequent upon hydrolysis by the sulphuric acid. Some proteids and vanillin also give a coloration.

Fehling's Test. The sections are steeped in concentrated copper sulphate solution, and after washing with distilled water are boiled in alkaline Rochelle salt solution (No. 97). The resulting cuprous oxide appears as black granules by transmitted light, and is most easily seen by dark-ground illumination. Glucose is the most usually indicated sugar.

Alcohol gradually precipitates inulin after some weeks, as spheroid crystals, which may be distinguished from calcium phosphate by dissolving them in boiling water.

Iodine-KI solution colours glycogen (e.g. in many fungi) dark red-brown.

Starches. Starch is found in the cells of all plants, except the fungi, in the form of small granules, which have often a special shape and size peculiar to any one plant; in addition, the granules found in several related species of certain natural orders are very similar in general characteristics. As the starch is a reserve material, it is found mainly in those parts of the plant which serve as storage tissue; such are the seeds, fruits, roots, rhizomes, and stems.

Commercially, starch occurs as a fine powder of varying shades of white. The experienced practical man can extract a surprising amount of information from the colour, taste, and feel. For microscopical examination the starch should be mounted and examined, first using a 16 mm. and later a 4 mm. objective. Mounting in water throws up the detail more clearly than dilute glycerine, but mounts in Canada balsam are best for polarized light work. Glycerine jelly is not of great value for the starches, since the mounting must be done warm, and the refractive index of the medium is not very suitable.

The starch is mounted by placing a little upon a cover glass, and tapping the underside gently with a pencil to distribute the grains evenly over the area. A drop of the mountant is placed on the slide, and the cover glass is inverted so that the starch is on the underside, and then lowered very gently on to the drop of mounting medium. In this way the mount is obtained free from air bubbles. A second method is to sprinkle the starch on to the drop of mountant, and after allowing a little time to elapse so that the material may sink under the surface of the drop, to place the cover glass in position.

Staining of the granules has its uses in certain cases, though usually it is unnecessary. It may be accomplished by mounting in very dilute iodine water, the grains becoming blue. Selective staining with iodine is accomplished by placing the cover glass, prepared with the starch as described, by the side of a watch glass containing solid iodine, and covering them up together for an hour or so, subsequently mounting in water or balsam. Methylene Blue, or a direct cotton colour, such as Chlorazol Fast Red K, may be employed for staining, this being best done in a small watch glass, taking out a little of the cream on to the slide for observation. As small a quantity of dye should be used as possible.

The points to be noted are the general shape of the grain, its thickness (this can be measured by means of the fine adjustment), length, and breadth, size of the largest and smallest granules, average size, markings, position of the hilum, and presence or absence of compound grains. A detailed summary of the reactions of several hundred starches is to be found in *The Differential Analysis of Starches*, by J. B. McNair (Field Museum of Natural History, Chicago, Publication 275).

Size. The size varies very greatly, from 1μ to 15μ. The length is the most important for identification, the breadth being only occasionally useful, though the relation of length to breadth is important. Certain starches contain more than one kind of granule, quite often a number of large granules, together with many which are much smaller, no intermediate size being present. Some starches never exceed a certain size, whilst other and very similar starches may have a larger limit of length.

Shape. Every starch has a certain definite and constant type of shape, which is rarely departed from sufficiently to make confusion possible. Some starches, it is true, may be easily confused with each other (e.g. wheat, rye, and barley), but in the majority of cases the shape and size alone are sufficient to enable the starch to be identified.

Markings. In many starches, such as potato, there is a point to be seen which seems to serve as a centre for concentric markings, like the contour rings on a map.

This point is termed the *hilum*, and is usually situated near the narrow end of the grain; the position is often very constant, the ratio along the long axis of the granule of its distance from the nearest margin, to its distance from the farthest margin, being very characteristic, and known as the eccentricity of the hilum.

The hilum in many cases develops a crack or hole, which takes certain shapes in certain starches, e.g. star-shaped, long, or branching. This crack is probably due to the bursting of the outer coat of the granule, and it is at this point that the grain bursts when swelling is induced by suitable means, e.g. hot water or phloroglucinol-oxalic acid mixture.

The contour lines are termed *striae,* or striations. They are not always present in all kinds of starch, but when present it should be noted that in most starches these are of equal intensity, only in a few varieties a difference in strength being noticeable. The striae are more probably due to differences in the refractive indices of different layers than to actual ridges on the surface.

Polarized Light Appearance. The characteristic appearance which is exhibited under polarized light takes the form of a dark cross on a light ground. Its great utility lies in detecting small admixtures of starch with other powdered substances, but it should be borne in mind that other substances, such as hesperidin crystals, may very occasionally produce this effect.

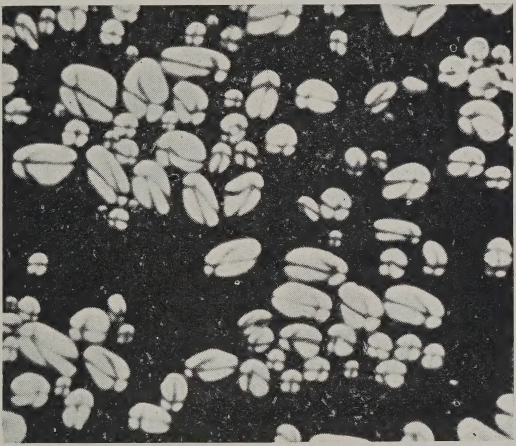

FIG. 132. POTATO STARCH UNDER POLARIZED LIGHT

Gelatinizing Agents. Many reagents possess the property of gelatinizing starch granules. Water at a temperature which is constant for each starch variety, causes the starch to burst at the hilum, the contents then swelling greatly and becoming translucent. This may be employed as an aid to identification. A thin cream is made on the slide in one drop of water, and mounted under a cover glass. The slide is placed on a hot stage, and the temperature gradually raised, the slide being kept under observation. The temperature is controlled by placing small crystals of chemicals melting at 5° intervals, on the slide. If preferred, the heating may be done, more accurately, in a small tube attached to a thermometer bulb, a little being removed by touching the surface with a capillary tube at definite temperatures, and blowing the drop on to the slide for examination. Polarized light should be used, as a completely gelatinous granule does not show the polarization cross. The important temperature range is from 50° C. to 80° C. The temperatures at which the grains begin to swell, begin to gelatinize, and gelatinize completely, are noted. The following table is taken from Polleyn.

TABLE IV

Starch	Rupture Clearly Marked	Gelatinization Begins	Gelatinization is Complete
	° C.	° C.	° C.
Rye	45	50	55
Maize	50	55	62·5
Chestnut	52	56·25	57·7
Barley	37	57·5	62·5
Horse chestnut	46·2	58·7	62·5
Potato	46·2	58·7	62·5
Rice	53·7	58·7	61·2
Tapioca	50	62·5	68·5
Wheat	50	65	67
Buckwheat	55	68·7	71·2
Acorn	57·5	77·5	87·5

Flours. Wheat is the most largely used flour, but rye and barley are also often employed industrially, together with maize. The starch present should be investigated as described, but in some cases more valuable information may be obtained from the structure of such harder parts of the grain as have escaped mutilation during the milling process.

The flour should be sieved in a No. 80 sieve, and the residue examined. The presence of mites, moths, beetle or weevil larvae or pupae indicates probable deterioration by long storage. This residue is placed in a fine muslin bag, and kneaded under water. The turbid liquid is settled, the solid washed by decantation two or three times filtered, and air dried. The grains of starch thus obtained are mounted and examined. Flour made from damaged grain will show granules partially eaten away by enzyme action, and fungal spores may be in evidence.

The bag left from the kneading contains the harder parts of the grain, together with glutin. It is opened out, stretched over a watch glass, and scraped out into a flask. Acetic acid is added, and after standing for some time, the whole is poured into the cloth again; the kneading is then continued until the water leaves the bag clear.

The residue is now ready for examination, and consists of any characteristic hairs and other structures which may be present. These resistant parts may, of course, be isolated more rapidly by preparing crude fibre from 5 grm. of starch, or they may be obtained by boiling with 1 : 20 HCl for a few minutes until the starch is hydrolyzed, centrifuging after making alkaline with KOH, and finally washing. The action of diastase may also be employed.

The following scheme has been adapted from Vogl, Nährungs und Genussmittel.

Remains of Palae. EPIDERMIS. Long cells, wavy walls, broken by crescent-shaped, semi-circular or circular cells, singly or in pairs, which may or may not bear a hair. Barley or oat.

Remains of Pericarp.

EPIDERMIS.

Cells have 4–6 straight walls—	
Lateral walls thickened	Wheat
Walls little or no thickening	Barley, rye
Hairs and stomata present	Barley
Cells have wavy walls—	
Wavy lateral walls	Maize 180 × 15–30μ pitted Millet 30–120 × 14–30μ
Wavy transverse walls	Rice 60–75 × 7·4–24μ

HAIRS.

Conical, thin walled, 30–180 × 9–21μ	Barley
Slightly tapering, very long, straight	Oats
Walls wider than lumen, bent, 1,000μ long	Wheat
Walls narrower than lumen, straight	Rye

TRANSVERSE CELLS.

Intercellular spaces present—	
Single layer of cells:	
Thin walled, straight, little pitting, rounded ends	Rye
Loose, narrow, rather long	Rice
Two rows of cells	Barley
No intercellular spaces present—	
Single layer of cells:	
Thick walled, straight, pitted	Wheat
Cells lying obliquely to epidermis	Oats

TUBULAR CELLS.

15–30μ wide, thick walled, scattered	Wheat, rye, barley
3–5μ wide, numerous, packed tightly	Rice, millet, maize

Aleurone layer cells.

One row, thin walled, square	Wheat
One row, thick walled, radially longer	Rye, oats, maize
One row, thin walled, tangentially longer	Rice, millet
One row, thick walled, tangentially longer	Buckwheat
Two or three rows	Barley

Tannins. Osmic acid rapidly forms a brown-black precipitate which is bleached or dissolved by hydrogen peroxide.

Iron salts: an etherial solution of ferric chloride is better than an aqueous solution, because the latter, being acid as a rule, gives a greenish colour.

Copper salts: the material should be steeped for some days in copper acetate, and washed. Sections are then treated for a few minutes with ferric acetate. The copper alone gives a brownish precipitate which changes to blue-green with the iron.

Alkaloids. Phosphomolybdic acid (10 per cent) precipitates many proteids as well as alkaloids. Alkaloids may be extracted by means of tartaric acid (5 per cent in alcohol) for a few hours. (For identification see bibliography to Chapter VIII.)

Proteids. Millons' reagent on gentle warming produces a brick-red colour action.

Biuret's reaction is uncertain for proteid matter in cells.

Iodine-KI gives a yellow or brown colour, though much less easily than with starches.

Concentrated HNO_3 gives a yellow colouration, made orange-red by alkalis. Many other bodies also give this colouration (tyrosine, resins, some oils, some alkaloids).

Globoids. Fat should be extracted, leaving calcium oxalate and globoids. Polarized light gives the starch cross with calcium oxalate which is strongly doubly refractive, but not with globoids, which are isotropic. Globoids do not always behave alike, but are usually soluble in KOH (1 per cent) and in 1 per cent acetic acid.

Resins. Copper acetate, acting for several days, gives a bright green with resin. The excess reagent should be well washed out before cutting sections.

Alcannin stains resins red.

Mucillage. Iodine-KI stains yellow to brown. Ammoniacal copper oxide effects partial solution. Water causes swelling. Corallin soda stains red. Indian ink does not penetrate mucillage. There are many varieties met with, which behave variously: thus some are largely soluble in water.

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CHAPTER XI

TEXTILE AND PAPER-MAKING FIBRES

For everyday routine work, dilute glycerine will be found very suitable as a mounting medium, as it gives slightly better contrast than glycerine jelly, due to its refractive index. On the other hand, it is so easy to mount a fibre in glycerine jelly, and the mounts are so permanent if thought worth preserving, whilst the slide is so readily cleaned if the mount is of only passing interest, that this mountant is entirely to be recommended. Acetate silk should be mounted, however, in water or Canada balsam, as it is rather indistinct in the jelly. If it be intended to take photographs by dark ground illumination, or if minute structures are to be examined by this lighting, then Canada balsam should be preferred above the others.

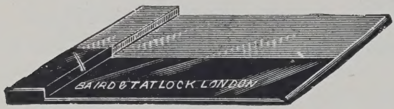

Fig. 133. Examination Plate

The mount should always be examined in a general way by means of the 16 mm. objective, in order to obtain a rough idea of the fibres present, the proportion, and probable nature, and subsequently a closer examination should be made with a 4 mm. It is totally unnecessary to employ higher power objectives for analytical work, since, apart from their well-known disadvantages, a more accurate idea of an object is obtained with a low power.

A point worth noting is that by choosing the mounting medium certain fibres may be rendered almost invisible.

Thus in a mixture of acetate silk with another rayon, the examination of the latter is considerably facilitated by mounting in dilute glycerine, which causes the celanese to become invisible in an almost magical manner.

Silk and rayon. The fibres which come into consideration are saponified and unsaponified acetate silk, viscose, undesulphurized viscose, cuprate, and true and wild silks.

Colour reactions are not of great value, because the majority of goods to be tested are dyed. Chemical tests are also to be used with caution, and are of value mainly as supplementary tests. The greatest reliance should be placed on the microscopic appearance, and in cases of doubt, preference should always be given to its indications of the appearance of the yarn and its cross-section over chemical tests. Parallel tests on type samples, and comparison with mounted standard fibres are absolutely essential, except in simple cases.

SILK. True silk under the microscope appears as a very regular glass rod-like fibre, in diameter about 12·5μ. This measurement is of importance, as even the finest rayons on the market are usually at least twice this diameter. The diameter may be read off instantly by means of an eyepiece scale previously calibrated, and graphed. There are now rayons which are equally as fine as silk. Silk in cross-section shows almost a true circle, with a strong tendency towards a triangular form, though in rare cases a poor quality of silk is seen to have a flattened section approaching that of a wild silk, but with no evidence of its multiple structure. Raw silk is, of course, a double thread, the two threads being cemented together by a rough coating of gum.

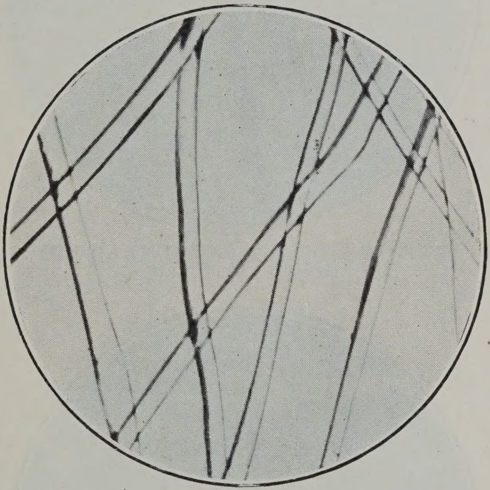

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FIG. 134. TRUE SILK (BOMBYX MORI)

WILD SILKS. Such silks as tussur appear like a thin flat ribbon, the breadth of which may be as large as 120μ in rare cases; striations such as can be seen on a blade of grass run lengthwise down the fibre. The general appearance of many kinds of rayon is rather similar at first glance, since they also are inclined to be flat or oval in cross-section, and exhibit longitudinal markings. Some of the wild silks are very difficult to identify as such by reason of this, but it will be noticed that always the longitudinal markings on the real silks run for a short distance and then disappear. The tussur fibre is really a bundle of from 10 to 20 separate fibrillæ, firmly cemented together, and it is this which causes the striations. The fibrillæ run along the surface for some distance, and then give place to others (true silk also has this structure, but on a much finer scale, and it

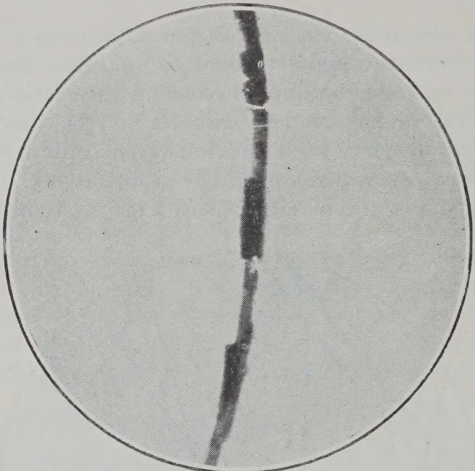

FIG. 135. WEIGHTED SILK, THE WEIGHTING FORMING A SHEATH ROUND THE FIBRE

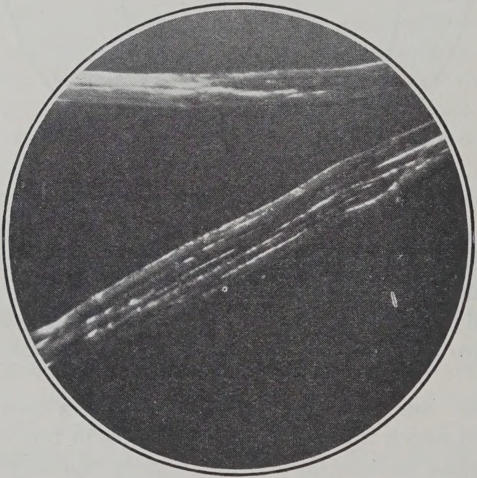

FIG. 136. TUSSUR SILK, UNDER DARK GROUND ILLUMINATION

is only after special treatment, and under high magnifications, that it can be seen). This structure must not be confused with the double

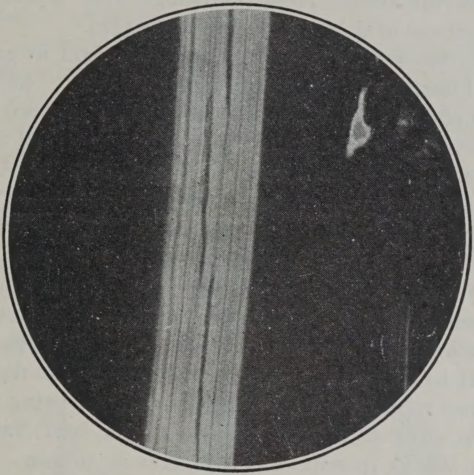

Fig. 137. Tussur Silk, under Polarized Light

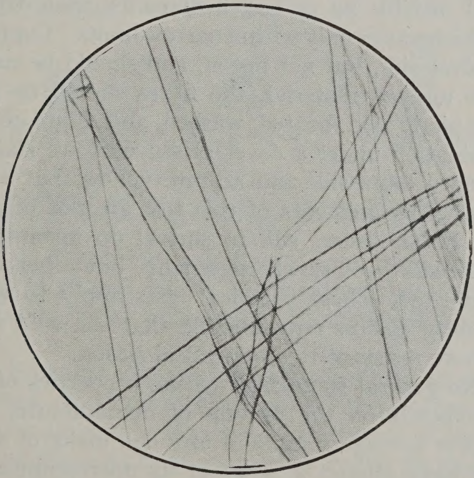

Fig. 138. Tussur Silk, under Transmitted Light

thread from the cocoon, for wild silks have little or no gum such as true silk possesses.

The artificial fibres, on the contrary, owe these markings to the uneven shrinkage of the fibre substance on precipitation or drying, and the markings may often be followed for great distances along the surface of the fibre. These markings may usually be better observed by means of dark ground illumination.

Tussur in cross-section is a very flat oval, and in good sections the individual fibrillæ may be easily distinguished. The fibrillæ may also be observed in places where the fibre has been torn at the ends, and as tussur is usually employed in spun silk fabrics, a number of these places may be observed in any average mount. A further and important characteristic of true and wild silks is that in many places there is a diagonal flattening of the fibre, where threads have crossed in the cocoon. These markings show up very clearly in polarised light, and are never, in the author's experience, to be found in rayon.

To distinguish chemically between true and wild silks, concentrated sulphuric acid is an excellent reagent, wild silks being much more resistant to its action. Concentrated HCl also dissolves cultivated silks very rapidly, wild silks such as tussur being only slightly attacked even after 48 hours. Boiling 10 per cent NaOH solution dissolves true silk in 10 min. and wild silks in 30 min.

In addition to the tests already given, the following notes will be useful. In some cases rayons are found which have no striations, but they will usually be of larger diameter than true silk, the diameter of which varies only within narrow limits. Copper glycerine solution dissolves silk, but not rayon, and should be used on dyed material. For undyed material, the fibres should be placed in a drop of dilute picric acid, heated, washed, and dilute glycerine used for the examination under a cover glass; silks are stained yellow. Iodine solution colours silk and acetate yellow, but other rayons brown or violet. Refinements of this test are not of great value. Concentrated HNO_3 causes silk to shrivel up instantly and turn brown; acetate silk goes slowly to a jelly, but other varieties are not visibly affected. These chemical tests apply to wild silks as well as true silks, always remembering that the wild varieties are often much more resistant to all chemical action.

RAYONS. No general summaries of the properties of rayons are safe. Huch depends on the process of manufacture, and during recent years the average sample of any one make of artificial silk will certainly have altered in some of its microscopical characteristics, due to improvements in manufacture. Thus, viscose silk was at one time often found in the form of a smooth rounded fibre, but many makers now turn out a fibre with a strongly corrugated cross-section, as this is supposed to give certain advantages. Again, two viscose fibres from different manufacturers will show differences in cross-section, and, in fact, the cross-section is the most reliable guide at the present time to the maker. In consequence, the following remarks should be taken as true in a general sense, but individual variations should be expected.

Cuprate. A glass-like fibre, with rounded cross-section, showing smooth edges. On the whole, cuprate is less lustrous than chardonnet and viscose, and has a metallic sheen. It is also often inclined to be harsh and dry to the touch. Very fine filaments may be made by this process, and at the present time an artificial silk filament of about the same size as true silk should be suspected to be this variety. Various chemical tests have been proposed, based on the traces of copper which are usually present, but only the sulphuric acid one is at all reliable. Cuprate silk has a very low copper index.

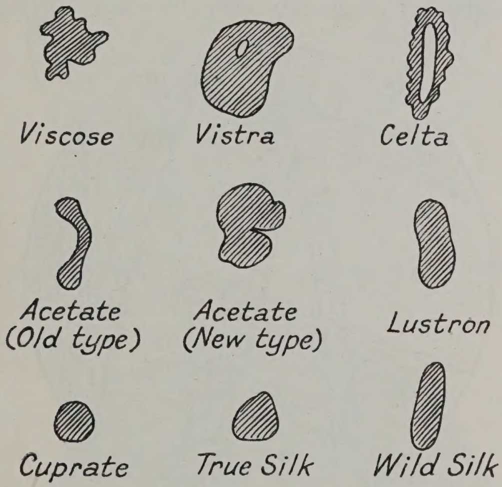

Fig. 139. Cross-sections of Artificial Silks

These diagrams are only accurate at the present time, as cross-sections are constantly changing with variations in manufacturing methods

Acetate. Probably acetate silk is the nearest approach to true silk yet devised. It is warmer to the feel than other rayons, and has a peculiar soft lustre. It is a non-conductor of electricity, like true silk. Very fine counts of spun yarn should be suspected to be this material, as it can be spun to finer counts than most others owing to its peculiar cross-section. It is very resistant to sea water and other media containing bacteria. An excellent means of distinguishing it from other rayons is provided by a 40 per cent solution of KI, specific gravity 1·3, in which it floats, in common with true silk, other fibres sinking. Not a great amount of acetate silk is saponified at the present time, the dyeing difficulties of the unsaponified material having been largely overcome. The two modifications present exactly the same appearance under the microscope, but glacial acetic acid dissolves the unsaponified fibre and iodine stains it yellow; the saponified fibre is undissolved by acetic acid, and generally behaves like viscose. The appearance under the microscope is very distinctive, and is best seen from the cross-section. Some foreign brands are more akin to viscose in appearance.

Viscose. The cross-sections of most makes show a very angular corrugated outline. The general tendency to-day of all rayons seems to lie in this direction. It has a very high lustre. By far the most common type of rayon, it does not often occur in very fine filaments. Many varieties of viscose, chiefly of continental origin, contain numerous air bubbles. Vistra is one of the best known of this type.

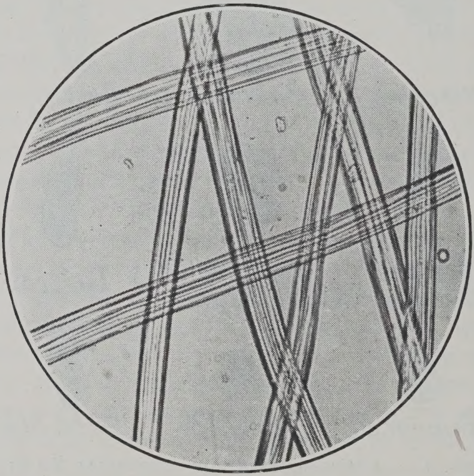

Fig. 140. Viscose Rayon

For identification purposes, the cross-section is extremely important, and gives very reliable results. With undyed yarn, the dyeing affinity is also a good guide, and a little can readily be dyed in a drop of water together with some known viscose, the results being useful as an indication of the identity or otherwise of two samples, though (in the early days much more so than now) even batches from the same manufacturer may take the dye very differently. The solubility in caustic soda is also of great value, though it cannot be applied as a microscopic test. The twist per inch, readily ascertainable by microscopic examination under a low power, varies with different spinners and makers, but may occasionally be useful. A more valuable indication is the denier of the individual filaments, or, what comes to much the same thing, the number of filaments in

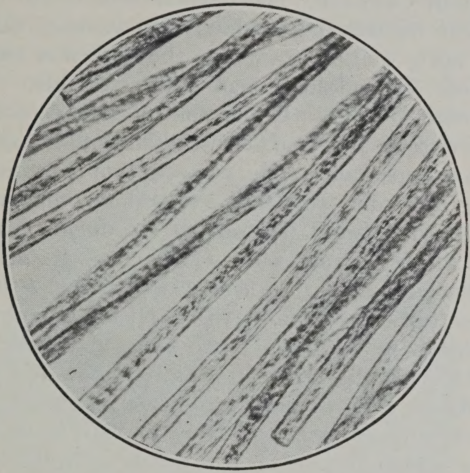

FIG. 141. VISTRA RAYON BY TRANSMITTED LIGHT, SHOWING AIR BUBBLES IN THE FIBRE

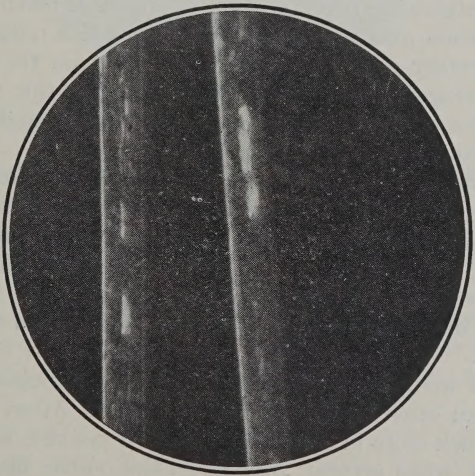

FIG. 142. VISTRA RAYON UNDER DARK GROUND ILLUMINATION, SHOWING AIR BUBBLES

any given denier. If a whole hank is available, much information may be obtained from the lace bands and method of lacing.

VISCOSE FROM CUPRATE. The cross-section is the best test, for cuprate is more regular and rounded than viscose; this distinction may be seen fairly well by examining the broken or cut ends of the fibres mounted. The sulphuric acid test is valuable.

VISCOSE FROM ACETATE. Under the microscope acetate is very distinct from viscose, as it bears a resemblance to a magnified cotton fibre in general appearance. The cross-section is extremely characteristic. There are many sure tests available for this differentiation, the specific gravity being the safest. Its dyeing properties, and solubility in many organic solvents, such as glacial acetic acid, acetone, aniline, or cresol; the colour with iodine; the nitric acid jelly: all these tests are characteristic, and may be applied according to circumstances. It burns quite differently from viscose, and on heating it by passing a thread rapidly backwards and forwards in a bunsen flame, it grows plastic and easily breaks.

VISCOSE FROM SAPONIFIED ACETATE. This is of little importance, but the specific gravity test is the most valuable.

VISCOSE FROM COLLODION. Collodion silk presents quite a unique appearance under polarized light, and this test should be employed.

The diphenylamine reaction so often given in textbooks is unreliable. Collodion silks contain oxycellulose, which reduces Fehling's solution, a rather good test when carried out on the slide with a drop of the reagent, but some cuprate silks give this reaction also. The silk is not of great importance to-day; it is found occasionally in continental cloths, particularly Belgian.

Animal Fibres. WOOLLEN, WORSTED, BOTANY. The chief difference between woollen and worsted yarn from the microscopist's point of view is that worsted yarns usually have the fibres more or less parallel, and hence are very easily teased out when preparing them for mounting. Mule-spun fibres, which have usually been carded, and in which the fibres are criss-crossed, are more difficult to tease out.

Unscoured wool shows adhering to it bits of grease and dirt, and the scales can often be only seen indistinctly. After scouring, the scales are much more in evidence. The fibre consists of three parts. The medulla, when present, runs down the centre, but it is mostly absent in good wools; its presence is usually a sign of low quality or coarse hair. It should be remembered, however, that close to the root of the hair it is almost always to be seen. The main bulk of the fibre is termed the corticle tissue, and is of no importance from an analytical standpoint. The outside layer, or epidermis, consists of flattened horny tubular cells, in the form of overlapping scales, which are most important for identification. Fine merino wools have these scales going almost round the fibre; in the finer qualities of wools the edges are apparently smooth, only under high powers showing serrations.

Fleece Wools. These are obtained from living sheep. The first shearing is lamb wool, and may be known by the tips of the fibres tapering to a blunt point, the scales gradually becoming less distinct as they approach this. Subsequent shearings, or wether wools, show this tip to be absent.

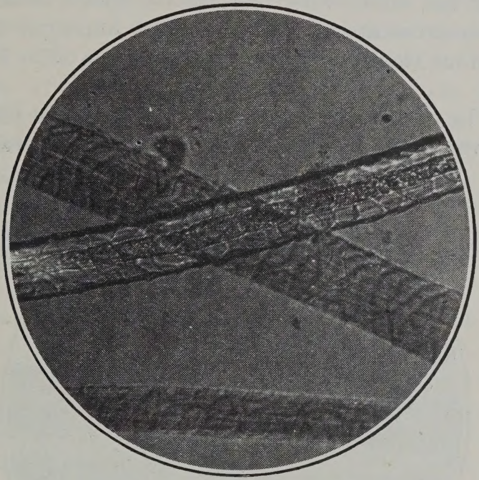

Fig. 143. Worsted Fibres

Skin Wools. These are obtained from dead sheep by means of depilatories. They are harsh and rough, and may have the scales damaged by the chemical action of the lime used.

Kempy Wools. These are distinguished by the coalescing of the scales. They are found in the neck and legs mostly, or on diseased sheep. They are impervious to water and, therefore, do not take up dye well.

Chlorinated Wool. A considerable weight of wool is now chlorinated. Treatment with hypochlorite solutions has the effect of partially removing the scales, and of rendering the wool less liable to felting. Over-chlorination removes the scales entirely, rendering the wool worthless. Damage to the protective scale coating may be detected by the Pauly reaction, which consists in treating the wool with diazo-sulphanilic acid solution, faulty places becoming pink.

Staple. The length of the hair may be obtained roughly by measuring the diameter of the fibre. The approximate counts are as follows—

Merino		80/60 counts,	15μ
Crossbred,	fine	58/50's counts (botany)	20μ
,,	medium	48/46's counts (South Down)	32μ
,,	coarse	44/32's counts (Lincoln)	60μ

Goat Hairs. Each variety of goat gives two kinds of hairs, a soft and more or less white wool hair, and a coarse beard hair. The diameter measurements are of the utmost importance in deciding between various closely similar fibres, and are often the only test available.

Mohair. In the trade to-day, mohair may mean either the true hairs from the Angora goat, or may apply to a mixture of the various goat hairs with worsted. The diameter of mohair is about 45μ and is very constant. The medulla in the majority of cases will be visually absent, but there are always thicker hairs present which may easily be confused with some goat hairs, though these have a larger diameter than the mohair beard hairs. The medulla sometimes becomes visible in polarised light, even when apparently absent under the usual lighting conditions.

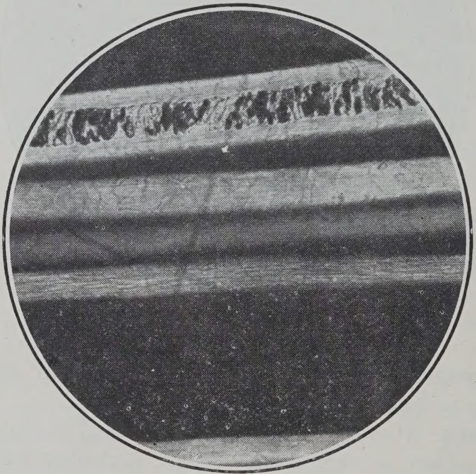

Fig. 144. Mohair Fibres under Polarized Light

Running along the length of the fibre are numerous markings, as though the hair had crinkled up. These are a very good distinguishing mark between mohair and worsteds. All sheep wools have similar markings, but they are always much less prominent or almost invisible.

The scales on mohair are shorter than those on wool, and often surround the whole fibre. They are very flat and in some cases it is difficult to see them at all. The edge is often very finely serrated. It is difficult to distinguish with certainty between a low quality of mohair, and certain grades of worsted, and in some cases the only test of value is to turn the fine adjustment to throw the scales out of focus. With almost all mohairs, a mere touch will suffice, but worsteds require a greater amount of turn of the milled head.

CASHMERE. The diameter is an important point in the recognition of this fibre. It comes on to the market in three or four main qualities, which may be considered as mixtures of two kinds of hairs, one fine, soft and white, with a diameter as fine as silk, 12μ, the scales, however, quite distinct, and medulla mostly absent. The other type is quite coarse, brown in colour, with an average diameter of 70μ, the medulla very pronounced, and the crinkles of mohair very prominent.

ALPACA. This name covers many materials, and mixtures of mohair, worsted, goat hair, and cross-bred wool are so-called. Fine alpaca is also known in the trade as vicuna, though not often at present; vicuna more often denotes a mixture of wool and cotton.

True alpaca may vary in colour from white through brown to black, but is very easily recognized by the peculiar medulla, with its black granular contents. There are two chief varieties of the wool hair, one white, the other brown; average diameter 20μ, with very fine scales, which can sometimes hardly be seen at all. The longitudinal crinkles are more conspicuous on the brown than on the white alpaca, though this may be an optical effect.

CAMEL HAIR. This fibre is now used quite largely because of its great softness; it is always highly coloured, and cannot be bleached successfully. The wool hair is about 20μ diameter, but the beard hair is often four times this thickness. The medulla is rather distinctive, and will in most cases be sufficient to differentiate it from alpaca, though this is sometimes not easy.

COWHAIR. This fibre is used on the continent for cheap carpets. It has no lustre, and the root of the hair may often be found. It may easily be confused microscopically with camel beard hair, but it has a wider medulla and thinner cell walls and the diameter may be up to 150μ, whilst camel beard hair averages 75μ. There are also numerous scales present. Fine cowhairs are rather similar to sheep wool, but by warming with 10 per cent KOH there is a distinct difference observable in the method of disruption of the corticle tissue, though this difference is not so marked between cowhair and goat hairs.

Other hairs occurring commercially are horsehair, rabbit hair, and cat hair, which are easily recognized from type samples, and are only rarely met with.

Vegetable fibres. JUTE, HEMP, ESPARTO, FLAX, AND RAMIE. Some of these fibres are of more importance in the paper and carpet trades than in the textile industries. They are all composite fibres, and the structural characteristics of the fibres dealt with here are easily recognized. The minor seed hair and bast fibres are described in detail in Matthew's well-known book on the textile fibres.

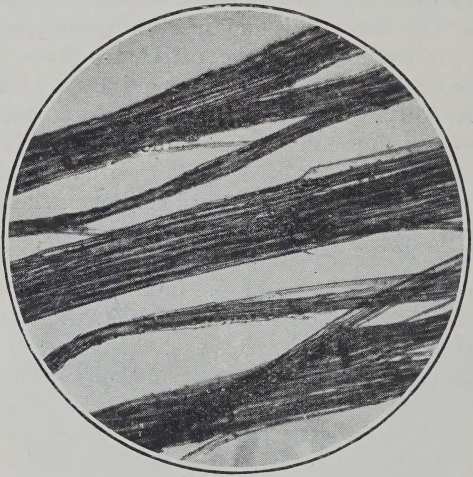

FIG. 145. JUTE

FLAX. The filaments show occasional lumps like knuckles, and cracks or dents in the sides. The ends are pointed. The chemical reactions are like those of cotton, and no reaction for woody tissues is given.

JUTE. The chief peculiarity of this fibre is that the lumen or central canal is very much narrower in some places than in others, owing to the variation in thickness of the cell walls. As a contrast to hemp, it has no knuckle markings. Chemically it is a lignocellulose, giving a yellow colour with iodine and sulphuric acid, a golden yellow with aniline sulphate, and a red with phloroglucinol. It readily dyes with basic colours.

RAMIE. Some very characteristic angular swellings occur on the fibres, which have also blunt ends. The diameter is very regular, but knuckles are often found and heavy fissures appear lengthways in the surface. The lumen is very broad, and polarized light often shows up deposits in it. Iodine-sulphuric acid gives a blue colour, and aniline sulphate none.

HEMP. On this fibre there are frequent joints and striations. The ends of the filaments are often bluntly forked, whilst flax never shows this peculiarity. The appearance in polarized light is rather important as a distinction from flax. Chemically it is slightly lignified, and hence its reactions (unbleached) are half-way between those of jute and ramie, but when bleached it behaves like ramie.

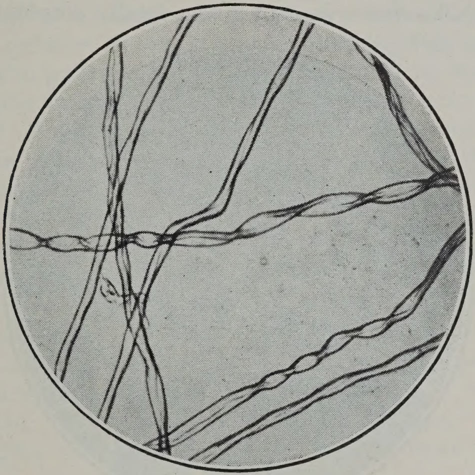

FIG. 146. COTTON BY TRANSMITTED LIGHT

Cotton. The appearance of this fibre under the microscope is well known and there is no textile material which is so easy to recognize. A few of the more important points are dealt with below.

In fully ripe fibres, cotton has a twist of 200 to 300 per inch, and the higher the grade of the cotton, the more regular and numerous is the twist. Fibres having little twist have usually also thin cell walls. The twist should extend close to the tip and base of the fibre, as length of staple is not more important in spinning than the amount of twist and the distance which it extends along the fibre. The depth of dyeing seems to be greatly influenced by the thickness of the walls, and faults in dyed pieces may sometimes be traced to this. Sea Island cotton has comparatively thin cell walls, and dyes less deeply than the coarser American and Indian cottons; whilst "dead" cotton, which has very thin cell walls, hardly dyes at all. It may also be mentioned that the turns per inch of the yarn may have a distinct effect on the depth of shade, and this can be determined microscopically.

"DEAD" COTTON. Twist is absent, the cell walls are very thin, the lumen is collapsed and almost invisible, and the fibre is like a twisted tape.

UNRIPE COTTON. This has little twist, thin cell walls, and rather indistinct lumen.

Both "dead" and unripe cotton have little cuticle, and hence when treated with Schweizer's reagent give a totally different appearance in the swelling produced. Iodine solution colours unripe fibres much paler than ripe fibres.

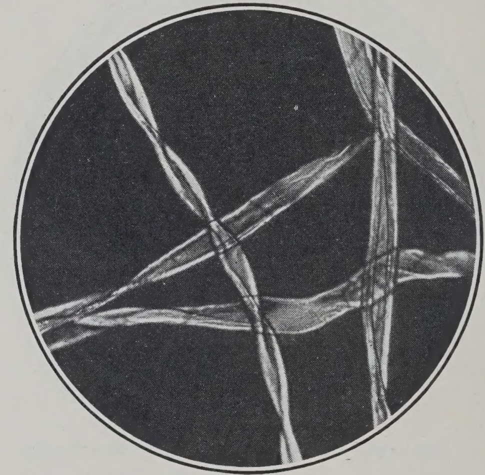

FIG. 147. COTTON BY POLARIZED LIGHT

NEPS. These are clusters of unripe fibres. These dye deeper than ripe fibres with direct-dyeing cotton colours, but less deeply with indigo and certain other dyes.

Many faults in cotton pieces may be traced to the presence of one or more of the above varieties of cotton by means of a microscopical examination.

MERCERIZED COTTON. In microscopical appearance this is quite distinct. Twist is apparently absent, and the fibre is swollen to an irregular glass rod shape. The cross-section shows this, and also makes it evident that the lumen has almost disappeared. The colour given with iodine is much deeper than that given by ordinary cotton, and is much more permanent to washing with cold water. A useful test is carried out as follows: A mixture of the sample with unmercerized cotton is dyed on the slide with benzopurpurine or Congo red and, after washing, is mounted in water under a cover glass. A drop of dilute HCl is placed at the edge of the cover glass, and flows gradually under the glass. If the unknown sample is mercerized, it will be bright red in colour under the microscope for some time after the ordinary cotton has changed to blue in adjacent places. Similarly, the test may be worked in the reverse direction.

Oxycellulose, which may be produced as a fault during many operations, gives a number of reactions which all depend on its reducing properties; hence, sizes and other substances which may be expected to interfere must first be removed by means of warm water. On treatment with iodine, oxycellulose goes blue, and on mounting in dilute sulphuric acid the colour is discharged. Cotton goes brown in the first place, and blue with acid. For dyed material, Fehling's solution offers a better test, since the cupreous oxide, which is deposited locally where oxycellulose is present, can be seen under the microscope.

Paper-making fibres. Strachan classifies paper fibres as follows—

1. RODS. Fibres of polygonal cross-section, with robust cell walls, large diameter, and regular lumen—

Hemp. Dia. 16–50μ, average 22μ. Lumen narrow, up to half the diameter of the fibre.

Linen. Dia. 12–30μ, average 18μ. Narrow lumen.

Manila. Dia. 12–40μ, average 25μ. Lumen more than half the diameter.

2. RIBBONS. Fibres of a flattened, ovoid, or prismatic cross-section, the cell walls robust or thin, the cell of great breadth—

Ramie. Dia. 22–80μ, average 65μ. Very robust cell walls.

Cotton. Dia. up to 40μ, average 22μ. Robust cell walls.

Hop. Dia. 22–30μ, average 26μ. Robust cell walls.

Coniferous Woods. (Spruce, larch, etc.) Dia. 20–70μ Cell walls of tracheids robust in summer growth, thin in spring growth.

Deciduous Woods (aspen, poplar) 20μ dia. Pitted vessels of over 100μ dia.

3. HAIRS. Fibres of narrow diameter, rounded cross-section, and narrow lumen—

Esparto. Dia. 12–20μ average 14μ.

Straw (common cereals). Dia. 5–20μ, average 12μ.

Phorium. Dia. 10–18μ, average 15μ.

Jute. Dia. 15–30μ, average 17μ. Polygonal cross-section, and variable but rather wide lumen.

4. FIBRES. The thin, oval, ovoid, or rectangular parenchymatous cells of straw, papyrus, and bamboo.

The following colour reactions are of value, but it must be emphasized that in order to obtain consistent results, the strength of the reagents, and the details of staining must be standardized.

HERZBERG'S IODINE (NO. 23A)—

Brown. Cotton rags, linen, bleached hemp.

Yellow. Mechanical wood pulp, jute, straw.

Grey-brown. Manilla, Adansonia.

Grey-white. Esparto, bleached straw, bleached jute, chemical wood pulp.

HERZBERG'S IODINE FOLLOWED BY SULPHURIC ACID (NO. 23B)—

Violet to Wine Red. Cotton, linen, bleached jute, esparto.

Blue to Grey. Chemical wood pulp, straw, esparto.

Gold to Brown. Mechanical wood pulp, jute, manila; overstained cotton, linen, bleached jute.

HERZBERG'S STAIN (NO. 26A followed by No. 26B).—

Reds. Cotton, linen, bleached hemp, bleached manila.

Blues. Chemical wood pulp, bleached straw, jute, esparto, Adansonia.

Yellows. Mechanical wood pulp, raw straw, jute, manila, esparto, ramie, flax, and lignified fibres in general.

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CHAPTER XII

INSECTS

Dr. L. O. Howard (formerly chief of the U.S.A. Government Bureau of Entomology) has stated that "the next big war for civilization may yet prove to be the struggle between man and insect."[1]

The annual loss due to the ravages of moth grubs alone has been variously computed at 22,500,000 lb. weight of wool (Dr. Meckbach) or 100,000,000 dollars (Better Fabrics League of America). This takes into account only wool and goods containing wool; damage to furs is to be reckoned in addition. The well-known ravages of the boll weevil in the American cotton-growing areas may be cited, whilst the damage to timber by wood-boring beetles, to flour and, indeed, organic matter in general, is almost incalculable.

Indeed, Major Walter Eliott, Chairman of the Research Grants Committee of the Empire Marketing Board, says: "One-tenth of all the world's crops are raised by man only to be eaten by insects. Everyone who buys a loaf of bread, a joint of meat, a pound of fruit, or a rubber tyre is forced to contribute towards the upkeep of the Empire's insects." The voracity of insects is extraordinary. Ealand mentions a plague of locusts that settled on a South African farm, and, in addition to devouring all the vegetation, ate also the washing hung out to dry on the line.

From our present standpoint, insects (using the term in the popular sense of the word) may be divided roughly into three groups: moths, beetles, and mites. The last group, of course, are not true insects.

This is not the place to describe methods for the prevention of the activities of these pests, but a brief summary of the conditions which are unfavourable to them may be of use.

Plenty of light, fresh air, good ventilation, cool temperatures are all effectual. Cold storage, sterilization (e.g. by means of steam), and fumigation (e.g. by mixtures of ethylene oxide and CO, or by means of siliceous earths saturated with HCN) have their uses.

Access may be prevented by special containers; or rendered less probable by the presence of camphor, naphthalene, p. dichlor benzene, pyrethium, and other substances obnoxious to insects.

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[1] This bold opinion is only one expression amongst many of the unsecure position of man in Nature. A prominent bacteriologist recently assured the writer, *apropos* of bacteria, that "they'll get us eventually!"

Material may be rendered immune from attack by impregnation, when practicable, with substances which act as poisons, such as creosote, fluorides, silico fluorides, alkaloids, cinchona, or the Eulan products.

Insects and parts of insects are conveniently treated in boiling 5 per cent KOH for a few minutes in a small evaporating dish. The heads, legs, etc., are removed on a needle-point, soaked in water for an hour, boiled in glacial acetic acid for 5 min., allowed to cool in the acid, taken out on a needle, and mounted in glycerine or glycerine jelly.

Mites require a less vigorous treatment, 50 per cent acetic acid being sufficient.

Staining may be done out of alcohol with chitin stains, such as picric acid or Congo red.

Moths. Three moths only are chiefly responsible for damage to articles containing keratin-like substances, such as wool and hair. The actual eating of the material is by the grub only; the moths have imperfectly formed and atrophied mouths.

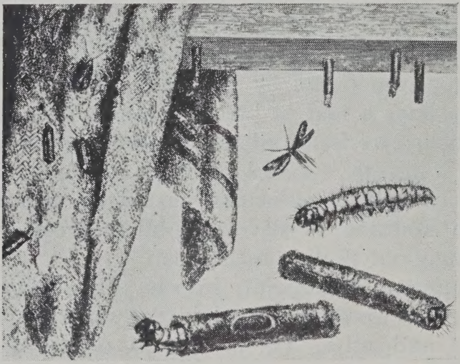

FIG. 148. THE TAPESTRY MOTH

Tineola Biselliella (the Clothes Moth). Pale gold, metallic lustre, wings covered with hair-like scales fringed with hairs. Wing expanse up to ½ in., rear wings slightly paler than front ones. The grub spins a silky tunnel as it moves about. When fully developed, a silky cocoon is spun in which bits of fibre are incorporated. One grub eats about 50–100 mgm. of fibre. Eggs up to 25 are laid at once, preferably in dark places, with no reference to the suitability of the material as food for the grub: white, 0·6 mm. long; 40,000 weigh 1 grm.

Tinea Pellionella (the Fur Moth). Head and forewings grey yellow or buff, showing indistinct darkish spots. Hind wings, white or greyish. All wings are fringed with long hairs. Wing expanse like T. Biselliella. Instead of spinning a tunnel, the grub builds round itself a case of silk covered with bits of fibre $\frac{1}{2}$–$\frac{3}{4}$ in. long. This is dragged along by the grub as it moves about, or is used as a shelter when resting. This case is sealed up prior to passing into the chrysalis state, thus forming a cocoon.

Tricophaga Tapetiella (the Tapestry Moth). Head and part of forewings black; rest of forewings cream, hind wings grey; fringed with long hairs. Wing expanse up to $\frac{3}{4}$ in. Grub, neither tunnel nor case is formed; the grub burrows in the material, lining the burrows (in which it lives) with silk.

Certain moth grubs are found in flour, amongst which may be cited Ephestia Kühniella Zeller, Corcyra cephalonica Stainton, and Borkhausenia pseudospretella.

Beetles. The dermestid beetles occur frequently and in widespread areas; their natural food is hair, sinews, skin, the more resistant portions of dead animals, and (strangely enough) many flowers. Under suitable conditions, however, they attack substances of industrial importance, such as wool, hair, leather, even beeswax.

The general characteristics are: Beetle, small covered with fine hairs or scales, which are easily damaged. The antennæ are short, eleven-jointed. The wing cases completely cover the body. Grub, variable in size, shape, and colour; usually hairy, active, and cast their skins several times during growth. No cocoons or tunnels are formed.

There are many beetles which attack wood, amongst which are the following: the furniture beetle, which attacks old furniture; the Powder Post beetles (hyctus brunneous and hyctus linearis), which attack timber; and the Death Tick Beetle, which is found in old roofs (Anobium domesticum).

Certain beetles, such as the Niptus hololeucus (cloth spider), are of common occurrence in cloth warehouses, especially where cotton is stored.

The silver fish is also often encountered, but seems to be of no industrial account.

Three beetles are frequently found in grain and flour: Calandra granaria Linn, Calandra oryzae Feib, and Sitodrepa panicea Thoms.

Many weevils are met with in very diverse situations, and may be recognized by their club-shaped antennæ.

Altogenus piceus olio (the Black Carpet Beetle). One-eighth inch long, black, as illustration. Eggs: white, oval. Grubs: finally

$\frac{1}{4}$ in. long, red brown, taper towards the tail, from which long hairs grow. They attack carpets, feathers, wool, and similar substances.

Attogenus pellio (the Furriers' Beetle). One-sixth inch long, fine black down on top, yellowish hairs beneath; three white spots near the end of the thorax; wing cases have reddish tone, and white dot in centre of each.

Anthrenus scrophulariae Linn (Buffalo Carpet Bug). Three-sixteenth inch long, black spotted, white speckles, red line down centre of back. Eggs: whitish, crinkled. Grubs: very active, shedding skin during growth, $\frac{1}{4}$ in. long, covered with long brown hairs; short tuft of hairs at tail. Attacks carpets, etc.

Anthrenus vorax (Woolly Bear Bug). Like a ladybird, but smaller; yellow brown, wing cases spotted with white and showing a broad yellow band. Eggs: white. Grub: very active, long legs, $\frac{1}{4}$ in. long, shedding skins during growth. Body shiny, cream, bearing dark brown hairs in transverse rings. Head same colour as the hair. Tail tuft with stiff pines at 45 degrees with horizontal. Occurs in Egypt and surrounding countries.

Anthrenus museorum (Museum Beetle). Oval, $\frac{1}{12}$ in. long variegated scales, brown to pale yellow. Wing cases show three irregular transverse bands. Antennæ eight-jointed, last two clubbed. Grubs: hairy, two tufts at the tail.

Dermestes vulpinus (Leather Beetle). Half-inch long, glossy, black, white hairs beneath, very active. Grub: black, covered with long erect hairs, up to $\frac{3}{4}$ in. long, burrows into timber for pupæ stage.

Mites (Acari). These organisms can be distinguished from true insects by their having four pairs of walking legs instead of three. Of industrial occurrence, the members of the family of Tyroglyphidae come into prominence; they commonly feed on decaying animal or vegetable matter. The Tyroglyphid mites are very small, soft bodied, and weakly chitinized. Some of them, like the remaining members of the Acari, are responsible for various kinds of "tick" and "itch" on human beings and animals, by burrowing into the skin. The following species are fairly often encountered—

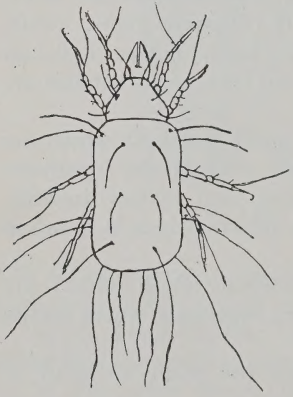

FIG. 149. TYROGLYPHUS LONGIOR

Tyroglyphus siro: the cheese mite.

Aleurobius farinae: the flour mite; occurs on grain, fodder, flour, and in cheese. The male has peculiar outgrowths on the front legs.

Tyroglyphus longior: occurs in grain, flour, fodder, etc. It is smaller than T. siro, more rectangular, and relatively longer.

Rhizoglyphus parasiticus: occurs in tea, and causes dermatitis on the coolies on tea plantations.

Glycyphagus domesticus: the house mite.

Glycyphagus spinipes: shows long feathered hairs.

Pediculoides ventricosus: the grain itch mite. Feeds on the pink boll worm, which occurs on cotton seed; occurs in grain and hay.

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CHAPTER XIII

MICROBIOLOGICAL TECHNIQUE

The unit of organic life is termed a cell, and consists of a mass of matter termed protoplasm, surrounded by a wall which probably consists of some form of cellulose. The wall is permeable to water and to substances in molecular solution, but within it is another layer consisting of protoplasmic jelly, which is semi-permeable. This semi-permeability depends on its structure, and its structure on its life; when the cell is killed, the inner wall becomes completely permeable to both electrolytes and colloids. This fact is the basis of certain tests using a colloid such as methylene blue, which will not enter a living organism.

The permeability of this membrane after death allows many substances necessary for the life of the organism to come out of the cell. Even during life the permeability varies considerably at certain times, but salt in solution can usually penetrate at any time, whilst sugars and certain high molecular weight bodies enter or leave with difficulty, except under certain conditions. In most cases, therefore, we do not know whether the characteristic reactions which the organism can bring about goes on inside the cell, or outside; thus, for example, yeast contains the soluble and low molecular weight enzyme, intertase, which may carry out the inversion of cane sugar after dialisis out of the cell; or, on the other hand, the sugar may pass into the cell, and its inversion products pass out. These facts are important in considering the suitability of an organism for industrial purposes, as if the product of the action of the organism is a low molecular weight substance, one may expect to obtain it in the reaction mixture whenever it is produced; but if a colloid like glycogen be the end product, it will probably be obtainable only from the dead organism.

Fermentation processes are usually carried out by the living organism, and hence the substance to be reacted upon should be capable of entering the cell. It follows that limitations are set upon the use of these organisms in chemical procedure, but it must be remembered that most of the effects produced by the living organism can also be produced by the dead cell without having first to extract the enzyme. In many cases, also, the reaction can be carried out by some inorganic or organic catalyst, the difference being that the living cell is more selective in its action, and is self-renewing by its growth.

The protoplasm contained within the semi-permeable membrane consists of a large number of imperfectly known substances, which are continually changing one into the other, with the result that there are permanent and transitory structures. One part of the cell usually is denser than the rest, and is termed the nucleus, which, however, is not important from the standpoint of this book. Many organisms have no nucleus, or are all nucleus, whichever point of view is preferred. It would seem that the nucleus bears a definite relation to the protoplasm, because as organisms get bigger, more nuclei appear. A nucleus surrounded by protoplasm is, so far as we know to-day, the fundamental unit of life.

The transitory structures are a result of chemical actions proceeding within the cell protoplasm; and when, as a result of these changes, the protoplasm passes from the liquid hydrosol to the gel state, a number of separate chambers (vacuoles), each with semi-permeable walls, are formed, containing a solution (cell sap).

It is thus quite possible for many apparently incompatible reactions to proceed at one and the same time in one organism of a diameter less than a thousandth of a millimeter

Roch, during his investigations on anthrax, found that at a certain stage of growth the bacteria formed a scum on the surface of the medium, and that at this point a denser granule grew in the middle of the bacteria. This condition is termed a spore. The rod form is the chemically active form of protoplasm, vigorously alive; chemical reactions proceed rapidly, and hence a large water content is present. The spore form, on the other hand, may be regarded as a storage form of life. Water is reduced to a minimum, the protoplasm is in a relatively dry state, and chemical changes go on very slowly.

Bacteria are considered to be very simple vegetable cells. They occur in three main forms: the bacillus, a rod-like form; and the coccus, a spherical form, and the spiral form. Some bacteria can appear as bacillus or coccus. Most bacteria are able to move about, at least at some stage of their life, and under suitable conditions. Reproduction varies with the organism, and may be by spore formation, or by fissure, the latter being more usual.

Sterilization.[1] The microscopical apparatus used in bacteriological work has already been largely dealt with. For the rest, the actual cultivation of the bacteria is carried out with much the same apparatus as is usually found in a chemical laboratory, with certain additions, which will be now described.

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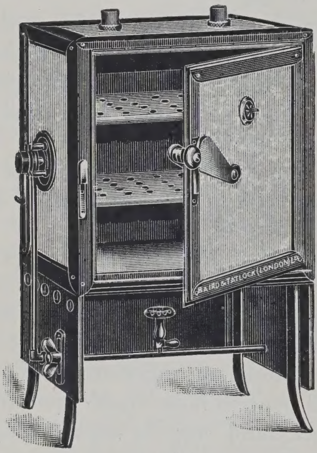
[1] It must be emphasized that any work on bacteria is attended by risk even under the best of conditions, when every precaution is taken to keep media and instruments sterile, and when the correct technique is employed to prevent any growth of bacteria outside the controlled conditions. In industrial work the organisms encountered will normally be quite harmless, but there is always the possibility that a pathogenic organism will make its appearance on a plate, and hence the greatest care should be taken at all times, especially in the sterilization of discarded plates, tubes, and mounts.

STERILIZATION APPARATUS. A *steam sterilizer* and a *hot-air sterilizer* are essential, as will be seen later; whilst an *autoclave* for sterilization with steam under pressure is very advisable, though not absolutely necessary.

The Hot-air Oven. This should be about 18 in. square inside, and should have a false asbestos-covered bottom at least 3 in. above the bottom of the oven. As the temperature has to be kept at 160° C., it is necessary to have a good flame underneath, and temperature control is facilitated by having a double wall, or an asbestos lining. The oven should be situated out of a draught. It is also necessary to have a thermostat, very convenient forms being the mercury and the capsule types.



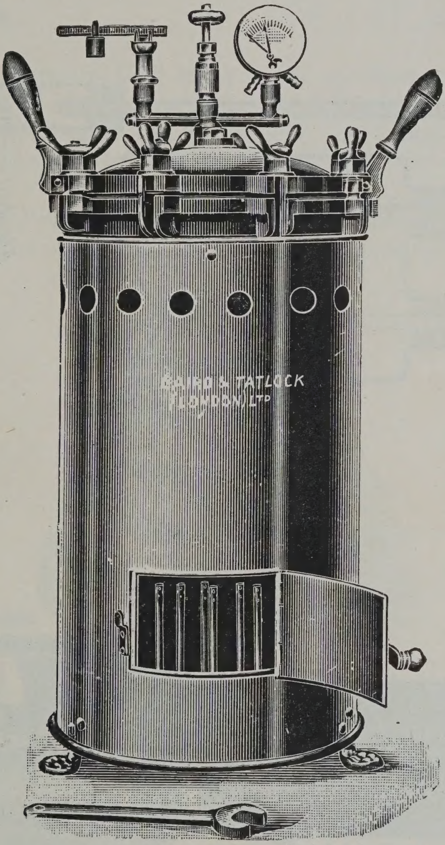
(*Baird and Tatlock*)
FIG. 150. HOT-AIR STERILIZER

For occasional work in a chemical laboratory, an excellent *steam sterilizer* may be found ready made in the double pans which are used for steaming vegetables. These consist of a pan which has, in place of a lid, a second slightly smaller pan with a perforated bottom; the top pan is, of course, fitted with a lid. The more elaborate types of sterilizer specially constructed for bacteriological work have a double wall, which conducts the steam back into the boiler, thus preventing the escape of unnecessary steam into the laboratory. It should be pointed out that "wet" steam is essential, as dry steam is a much less efficient sterilization agent.

Fig. 152 shows an *autoclave*, which is chiefly useful for the sterilization of resistant bacteria of the spore-forming type, and is also very convenient for sterilizing apparatus containing old cultures which are no longer required. The apparatus may be placed inside an iron pot (provided with a lid) and sterilized at 30 lb. pressure with moist steam for half an hour, after which it may be boiled up in water, washed, and cleaned for further use.

A further method of sterilizing old cultures is to add a quantity of 1 per cent mercuric chloride, afterwards heating the apparatus until the medium becomes liquid.

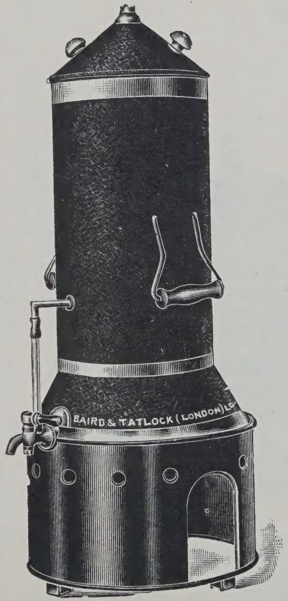
A container for sterile water is also required. This may be made from a thin-walled Wolff's bottle, with two necks, by fitting it with tubes as shown. The water is siphoned out as required. The lower end of the siphon tube is protected by being kept inside a small test tube containing alcohol. The water should not be considered sterile for more than a day.



(*Baird and Tatlock*)

FIG. 152. AUTOLCAVE

Incubation and Culture Apparatus. A hot-air oven for 37° C. is required fitted with a thermostat, and kept at a temperature which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(*Baird and Tatlock*)

FIG. 151. STEAM STERILIZER

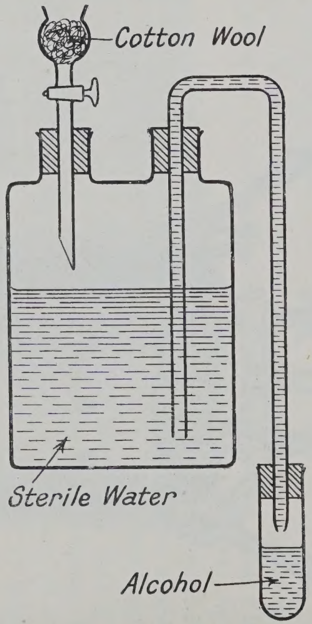

Cotton Wool
Sterile Water
Alcohol

FIG. 153. STERILE WATER CONTAINER

is decided by the type of organism to be examined. The temperature ranges used for various organisms may be summarized as follows—

Pathogenic bacteria	37° C.
Non-pathogenic bacteria	20° C. ± 2° C.
Yeasts	27° C. ± 2° C.
Moulds	35° C. ± 5° C. and 20° C.

It should be noted that there is an optimum temperature of growth for each organism, and that, in addition, the temperature at which growth begins and ceases varies with each organism. In some cases, selective growth can be obtained by controlling the temperature.

A supply of *Petri dishes* is essential, the most convenient size for general work, having an upper dish 4 in. in diameter. These should be kept in a suitable box when sterilized, out of reach of draughts and dust, but it is difficult to ensure complete sterility for more than a few days.

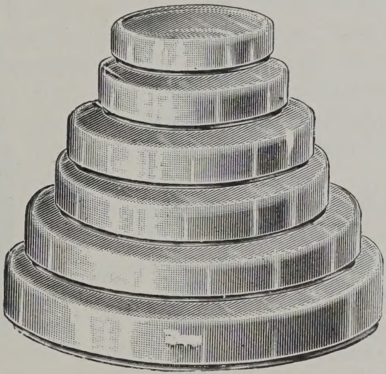


FIG. 154. PETRI DISHES

Smith's fermentation tubes are necessary for the determination of gas production and the rough analysis of the gas formed.

The *Durham fermentation tube* is easily made as required by inverting a small test tube inside a larger one.

Hansen flasks are also a great convenience.

All media used for the cultivation of organisms must be treated in such a way as to kill any living matter with which it may have become infected from the air or other sources; this applies also to all glassware and any instruments, such as platinum loops, which may come into direct contact with the media or cultures.

Once the sterilization has been effected, precautions must then be taken to ensure that no further contamination takes place during storage or during the incubation of an organism.

Three principal methods are in vogue for sterilization of media—

Pasteurization. The liquid is heated once for half an hour at 100° C. All non-sporing bacteria are killed by this treatment. Actually most bacteria of this class are killed by boiling for about 3 min.

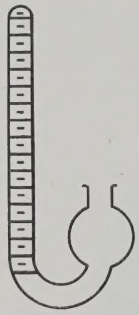


Fig. 155. Smith Fermentation Tube

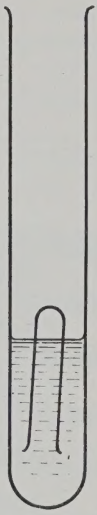
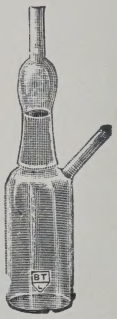


Fig. 156. Durham Fermentation Tube



(*Baird and Tatlock*)
Fig. 157. Hansen Flask

Tyndallization. Although spore-forming bacteria themselves are killed by Pasteurization, any spores which may be present survive, because they resist comparatively high temperatures with ease. The liquid is, therefore, kept at 20–30° C. for several hours to allow spores to germinate, and is then heated to 100° C. for half an hour to kill the bacteria produced. This procedure is repeated at intervals of not less than six hours, until the medium has been sterilized three times. The sterilization is usually done on three successive days.

Autoclave Sterilization. By heating to a sufficiently high temperature, spores may be killed by one heating, for no spore seems able to withstand a temperature of 120° C. in wet steam or in a liquid medium. Laboratory experience shows that 1 hour at least is necessary, using an autoclave at one atmosphere pressure.

Glassware is much more difficult to sterilize than liquids, for spores in the dry condition (and glassware should always be well dried before sterilization) are much more resistant. A temperature of about 160° C., which is the temperature at which cotton wool begins to go brown, is usually required for 1 hour. Higher than this temperature it is not advisable to go, for the cotton wool, which is used as plugs for flasks and test tubes, becomes brittle.

Platinum wires and loops may be sterilized immediately before and after use by passing them through a bunsen flame.

Glass flasks and bottles, and test tubes, should be well washed in water containing a little soda ash, boiled in a dilute solution of hydrochloric acid, and finally well dried. The neck is then plugged with cotton wool, which must be dry, for it acts as a filter from the air; and any mould spores settling on moist cotton would develop, rendering the "seeding" of the contents of the vessel likely. The plug should be so tight that the apparatus can be lifted by it, but should not be so tight that it is difficult to withdraw. It should not be conical, and a liberal amount of cotton wool should be used, so that there is a large bunch outside the neck.

When storing sterilized containers for any length of time, the plug should be cut flush with the neck and molten paraffin wax poured on. It is also a good plan to seal the neck by means of one of the viscose caps which are used by chemists for sealing medicine bottles. A filter paper folded round the neck and tied with cotton will also serve.

For storing apparatus and sterile media, a special room is advisable, free from draughts, and kept as clean as possible, in order to ensure the absence of dust. Tables should be washed down frequently, and occasionally methylated spirits used for washing. Apparatus should be cleaned with a sterilized cloth.

Petri dishes are difficult to keep sterile, and a good plan is to make them up into parcels with tinfoil, and place the parcel in a box with a tight-fitting lid, in order to keep away draughts which might cause reinoculation from the air.

Before any vessel is opened, the cotton-wool plug should be ignited and the flame blown out, in order to sterilize the exterior of the plug. Flasks and test tubes should be opened in as horizontal a position as possible, in order to minimize infection from falling dust. As an additional precaution, some work under a large sheet of plate-glass, the underside of which is kept scrupulously clean.

The room in which the work is carried out must be free from draughts, and all windows should be kept closed, in order to minimize the possibility of preparations becoming inoculated by airborn organisms.

Culture methods. The main objects of industrial bacteriological work may be summarized as follows—

1. To discover a medium upon which the organism will grow under controlled conditions.

2. To separate the organisms present into pure types and examine their suitability for the work under investigation.

3. To ascertain the reactions of the pure cultures for the purpose of identification.

4. To estimate the total number of organisms present in a definite volume or weight of the original substance, i.e. a quantitative estimation of the concentration.

5. To isolate and estimate the concentration of so-called "indicator organisms" when this is practicable.

6. To discover the best conditions for growth, or the most efficient means of repressing the growth, as may be desired.

There is usually a mixture of bacteria, moulds, and yeasts as the raw material. Their separation may be brought about by inoculating some solid sterile medium which serves as food for some and not for others; or by lowering concentration to a point at which the colonies produced by the reproduction of each individual organism are sufficiently distant from each other in the medium to enable small portions of the colonies to be picked out without fear of contamination by neighbouring colonies.

The solid media employed have in general a base of gelatine or of agar. The former serves as a food of the protein type, whilst the latter may provide cellulosic food: the important difference between them is, however, to be found in the melting point of the jellies. It is a simple matter to obtain an agar jelly which does not melt until 98° C. is reached (solidifying, however, at 40° C.); whilst gelatine jellies melt as a rule well below the temperature of 37° C., which is required for incubation at blood heat. Gelatine is, therefore, employed for incubation at room temperatures, whilst agar is used for higher temperatures. For industrial purposes, gelatine is largely used, though agar may with advantage be employed as a thickener for all media, for incubation at any temperature below 40° C. Agar is very rarely attacked by bacteria and the resultant growth is therefore entirely due to the constituents of the medium. This may be of importance, for example, in dealing with fermentation of sugars, since some organisms produce gas in sugar-free gelatine.

As a rule, to both types of medium, food is added in the form of meat extract, with or without some sugar, such as dextrose; other constituents may be incorporated for special purposes; thus indicators or chemicals which react with products of the growth of organisms enable acidity, the production of sulphuretted hydrogen, and the like, to be seen with ease.

The principal media employed are nutrient gelatine (No. 56), nutrient agar (No. 60), and nutrient broth (No. 52). Various other media for special purposes are described in Nos. 52–75, their use being indicated in the proper place. The pH of the medium is of considerable importance, and special attention should be paid to the method of adjusting the reaction (No. 51).

Filling tubes with culture media. The preliminary investigation of a source of micro-organisms is carried out in test tubes containing nutrient media. These are prepared in such a way that the medium is kept sterile during the transference from the storage flask to the test tube; and as the details of the process are of great importance, they will be given at perhaps greater length than might seem necessary.

(*a*) The storage flask containing sterile nutrient medium is placed in an incubator or hot-air oven until the medium is melted.

(*b*) A sterile test tube is taken between the thumb and first finger of the left hand, the storage flask being similarly held in the right hand.

(*c*) The two cotton-wool plugs are ignited in a bunsen flame and, after a moment, are blown out. (See Fig. 161.)

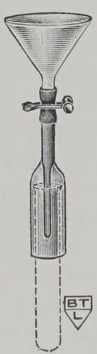
(*d*) With the crook of the little finger of the left hand, the plug is taken out of the storage flask; the plug is immediately withdrawn from the test tube in a similar way with the little finger of the right hand. The two plugs are held during the whole of the operation, and must not be allowed to touch anything.

(*e*) The medium is poured into the test tube (which should during the whole process be held as horizontally as possible) to a depth of about 1½ in., and the two plugs immediately replaced.

This method as described allows only one test tube to be filled at one opening of the storage flask. After a little practice, however, it becomes a simple matter to fill three test tubes in succession by holding the test tubes in the manner illustrated. The plugs are withdrawn by being gripped, one between the third and fourth fingers, the second and third fingers, of the right hand, and by the crook of the little finger. (See Fig. 162.)

This method has certain disadvantages, one being that the mouth of the test tube is gummed up with the medium, the result being that the plug sticks in the test tube; a more serious objection is, however, that the medium has quite sufficient opportunity to become contaminated by floating micro-organisms in the air.

When a number of tubes is required, a most useful method is to pour the melted medium into a sterile funnel fitted with a stopcock, the funnel being covered with a glass plate. Tubes can be filled very rapidly in this way, and with much greater certainty of the medium remaining sterile. The method is to be recommended whenever more than two or three tubes are required.



(*Baird and Tatlock*)
FIG. 158.
APPARATUS FOR FILLING TUBES WITH MEDIA

It is always advisable to sterilize tubes of medium thus prepared at least once in the steam sterilizer, after which they are allowed to cool and set. Agar tubes may all be set sloping at an angle of 20 degrees with the horizontal. Gelatine tubes should, however, be set, some in this position and some standing vertically.

Nutrient broth, and liquid culture media in general, are dealt with according to the above procedure, with the exception, of course, that there is no necessity to allow them to set solid.

Types of cultures. For the sake of simplicity, it will be assumed during the next few paragraphs that a liquid infected with micro-organisms is being examined. Details will be given later of the methods of dealing with various kinds of infected sources.

The four main types of culture employed are respectively designated streak, stab, shake, and broth cultures. The streak culture is designed to promote the growth of those organisms which require air, whilst the stab culture enables those organisms to promulgate which grow best when air is to some extent excluded. A shake culture is not employed as often as a broth culture, though the purpose of both is essentially the same, being an enrichment of the life from the infected source, rather than the isolation of individual organisms.

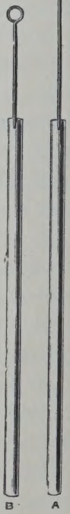


FIG. 159.
PLATINUM LOOP AND WIRE

STREAK CULTURES. (*a*) Sterilize a platinum loop by passing it through a bunsen flame.

(*b*) Dip the loop into the infected liquid under examination, and hold the loop in the thumb and fingers of the right hand.

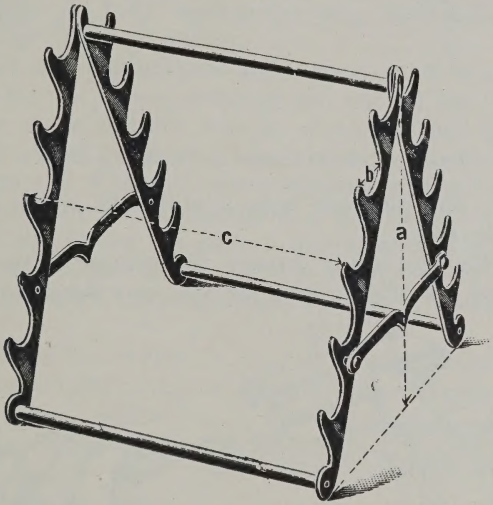
(*c*) Take a sterile agar slope tube in the left hand, ignite the plug, blow out the flame, withdraw the cotton-wool plug by the crook of the little finger of the right hand, holding the slope tube almost horizontally.

(*d*) Draw the loopful of liquid in the platinum loop firmly along the slope in the test tube, but without breaking the surface.

(*e*) Withdraw the wire from the test tube, replace the cotton-wool plug, and pass the wire through the flame.

(*f*) Rest the glass rod of the platinum loop on a rod rest so that the wire does not touch the bench top.

The platinum wire must always be passed through the flame immediately before and immediately after use. Too much emphasis cannot be placed upon this simple precaution against accidental infection from the air, and danger from possibly pathogenic organisms.



(*Baird and Tatlock*)

FIG. 160. ROD REST

In cases where a solid culture must be treated in this way, it will be found advisable to take a little of the culture and stir it into a drop of sterile water in a small sterile test tube, treating this liquid as described above.

STAB CULTURE. A gelatine tube which has been allowed to set in a vertical position (i.e. the medium is not sloping in the tube) is used.

(*a*) Sterilize a platinum wire by passing through the flame.

(*b*) Dip the wire into the infected liquid, and hold in the thumb and first finger of the right hand.

(*c*) Take the gelatine tube in the left hand, ignite the plug, blow out the flame, withdraw the cotton wool plug by the crook of the little finger of the right hand, holding the gelatine tube almost horizontally. The platinum wire is still held in the right hand during this operation.

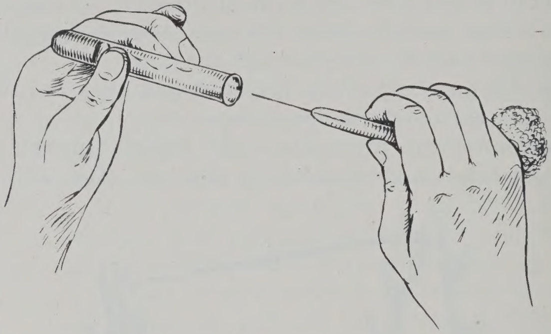


Fig. 161. Inoculation of a Single Tube

(*d*) Push the wire firmly through the gelatine to the bottom of the tube, once. On withdrawing the wire, care should be taken that the hole which has been made is not widened or torn. It should also be noted that the hole should close up instantly the wire is withdrawn; if it does not, the tube is too old for use, owing to the gelatine having hardened.

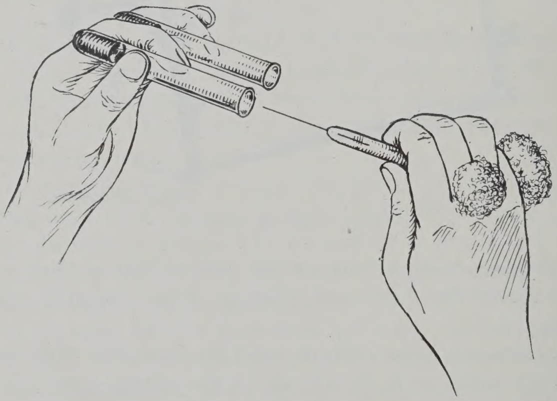


Fig. 162. Inoculation of Two Tubes

(*e*) Withdraw the wire from the tube, replace the cotton-wool plug, and sterilize the wire, replacing it on the rest.

Shake Culture. A tube of gelatine or agar is used; it should be melted, preferably by being placed in the steam sterilizer for a moment. It is then allowed to cool down to 40° C. in the case of agar (just above the setting point), or to about 30° C. for gelatine.

(*a*) Sterilize a platinum loop by passing through a flame.

(*b*) Dip the loop into the infected liquid and withdraw the loop, holding it between the thumb and finger of the right hand.

(*c*) Take the melted tube in the left hand, ignite the plug, blow out the flame, withdraw the plug with the little finger of the right hand, dip the loop into the liquid, and stir round once or twice.

(*d*) Withdraw the loop, replace the plug, and shake the tube gently to ensure thorough mixing.

(*e*) Sterilize the platinum wire and replace on the rest.

Broth Cultures. These cultures are carried out exactly as described for shake cultures, except, of course, that the medium being already a liquid, there is no need to melt the tube first.

Roll Cultures. A tube of melted nutrient gelatine is inoculated and is then held almost horizontally in a bath of cold water, being rotated until the gelatine has set in a thin layer on the sides of the test tubes. This culture is a convenient substitute for plating, at times, and also enables the under side of colonies to be observed very easily.

Hanging Drop Cultures. A drop of infected broth is placed on a cover glass in the centre; it should not be too large. The rim of the cavity in a hollow slide is smeared with vaseline and placed upside down exactly on the cover glass. It is raised carefully, and by a quick smooth movement is turned over into its correct position. It may be found difficult to focus the bacteria. This is best done by finding the edge of the drop with a low power and a pin-head condenser aperture, then swinging over the high power.

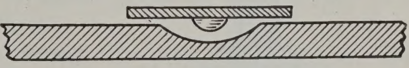


Fig. 163. Hanging Drop Culture

Plate Cultures. The methods of culture already described are of use chiefly in growing bacteria or other organisms on some suitable medium as a preliminary step, but it is obvious that no separation is effected, since all the organisms which may grow are mixed up one with the other. The method of separating the organisms present is known as plating. (See Fig. 164.)

It consists of diluting the bacteria present by means of a suitable solid nutrient medium until there is present, say, one organism per cubic centimetre. The diluted culture of the medium is then spread out in a dish so that 6 or 7 c.c. of the medium occupy an area of perhaps 12 sq. cm., that is, one organism is separated by a centimetre or so from its neighbour.

This single organism grows in its place, and after from 24 hours to several days, according to circumstances, several hundred thousand bacteria will have descended from it, all growing in one spot, and forming a "colony" which is visible to the naked eye as a little spot on the medium. As all these organisms have descended from one individual, they form a "pure culture" which is usually, however, tested by dilution and plating again in order to make certain that the rather unlikely coincidence of two organisms having grown together in the same spot has not occurred. It occasionally happens, however, that two organisms persistently grow together in this way.

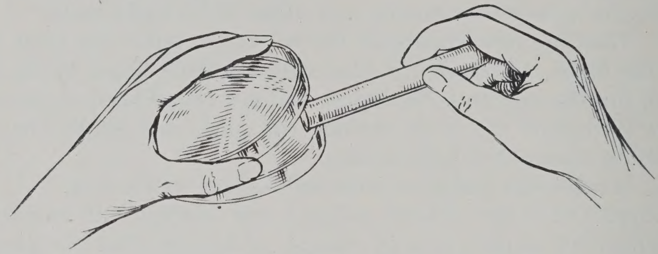


FIG. 164. PLATING

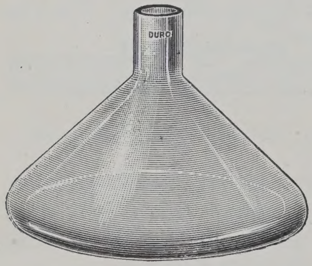


FIG. 165. CONICAL FLASK WHICH MAY BE USED IN PLACE OF A PETRI DISH

The operation is carried out as follows—

(*a*) Make a shake culture as described above.

(*b*) Place a sterile petri dish on the table and take the test tube containing the shake culture in the right hand.

(*c*) Ignite the plug and blow out the flame. Withdraw the plug with the little finger of the left hand, raise the lid of the petri dish with the left hand at one side only, and just sufficiently to enable the test tube mouth to be inserted.

(*d*) Pour the contents of the test tube into the dish and replace the lid. Replace the cotton-wool plug in the test tube, and as soon as possible spread out the medium in the dish by a gentle tilting movement.

Media, of course, take some time to set, and it is advisable to cool and set as soon as possible, which may be done by standing the dish on a slab of lead or other metal, or by standing it on a special plate cooler which contains running water. Media should be poured into the dish almost at their setting point, the low temperature minimizing the condensation of water on the petri dish lid.

A variation of this plating method is known as a *spread plate*. For this purpose a glass spreader is required, which is made out of a glass rod bent at an angle as illustrated. A petri dish is required, which has previously been plated with a tube of solid medium, and allowed to set. The advantages of a spread plate are that all the colonies are on the surface and easy to get at for further cultivation, and no confusion is caused by the same organism giving a different kind of colony on the surface and in the medium.

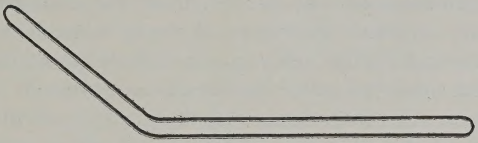


FIG. 166. GLASS SPREADER

(*a*) Sterilize a platinum loop, and dip into the infected liquid.

(*b*) Lift up the cover of the petri dish and streak the loop along one diameter of the dish. Lower the lid.

(*c*) Pass the previously sterilized glass spreader through a bunsen flame and, after again lifting the cover of the petri dish, stroke the liquid which has been introduced by the platinum loops evenly over the surface of the dish.

(*d*) Withdraw the spreader, close the dish, and sterilize the spreader by putting into methylated spirits, followed by steaming under pressure.

It should be noted that the methods of plating given assume that one dilution will be sufficient to separate the organisms from each other by plating. In practice this rarely happens, and in the case of strongly infected sources, such as sewage, much greater dilution is required. This may be done as follows—

(*a*) Take a loopful of the infected source, and add to 5 c.c. of sterile water in a small sterile test tube. Well mix.

(*b*) Take one loopful, and add to 5 c.c. of sterile water in a second sterile test tube.

(*c*) Take one loopful of the second test tube, and add to 5 c.c. of sterile water in a third sterile test tube.

Make a plate from each of these dilutions. The method may be continued to six or more plates as required. The dilution obtained in each successive plate is of the order of *x* 50. The final dilution after three plates is, therefore, about *x* 125,000 according to the method, the size of the loops and the contents of the tubes.

Counts of bacteria. It is often, if not almost always, of great importance to be able to state the number of bacteria present in the substance which is being examined. This is usually expressed as so many organisms per gramme, per cubic centimetre, or per million cubic centimetres. On the other hand, for certain purposes, the number of cubic centimetres containing one organism is stated.

In actual practice, as will be seen from the methods employed, the numerical valuation in either of these ways is only possible within certain wide limits; this question may conveniently be left at the moment until the procedure has been outlined.

SOLID SUBSTANCES (e.g. soil). (*a*) A 50 c.c. conical flask, or a Houston flask, is taken, and a definite volume of sterile water is taken.

(*b*) Weigh the flask and water accurately, add to it a quantity of from 1–10 grm. of the solid according to the case, and weigh again to obtain the exact weight of added material.

(*c*) Shake the flask and allow to stand for a few minutes, then shake again, allow to settle somewhat, and proceed as for liquids. (*N.B.* Not all the bacteria are dislodged, therefore return the count as the approximate number per gramme.)

LIQUID SOURCES. (*a*) Take three sterile 100 c.c. conical flasks, or Houston flasks, and measure out into each 90 c.c. of sterile water.

(*b*) By means of a 10 c.c. one-mark sterile pipette, add 10 c.c. of the liquid source to the first flask. Shake the flask, and close.

(*c*) By means of a second 10 c.c. pipette, take out of No. 1 flask 10 c.c. and add to the second flask. Shake the contents well.

(*d*) By means of a third 10 c.c. pipette, take out 10 c.c. of water from flask No. 2 and add it to the third flask. Shake the contents.

This process may, of course, be continued to whatever dilution may be required.

It is obvious that the dilutions are as follows—

Flask No. 1 . 10 per cent strength or dilution of × 10
,, No. 2 . 1 ,, ,, ,, ,, × 100
,, No. 3 . 0·1 ,, ,, ,, ,, × 1,000

In cases of highly infected material, such as sewage, dilutions of 1,000,000 or more are required, and this is usually done by adding 1 c.c. to 99 c.c. in a flask, in each case, instead of 10 c.c. to 90 c.c. as described above. The dilutions in this case are—

Flask No. 1 . 1 per cent strength or dilution of × 100
,, No. 2 . 0·01 ,, ,, ,, ,, × 10,000
,, No. 3 . 0·0001 ,, ,, ,, ,, × 1,000 000

Certain precautions must be taken if accurate results are to be obtained. It is of the utmost importance that the pipette should be drained as much as is reasonably possible, in order to get out the last drop of liquid, because in the case of a liquid which contained, for example, one organism per 10 c.c., it might happen that this organism was in the drop remaining in the pipette, and no result would, therefore, be obtained from this dilution. On the other hand, in the case where perhaps 100 bacteria were present per 10 c.c., assuming these to be evenly distributed in the liquid, the drop remaining, representing perhaps 0·2 c.c., would contain one-fiftieth of the total bacteria, and the next dilution made would be, therefore, in error to that extent. This error is cumulative and grows larger with each dilution. In very accurate work an allowance should be made for this, which is estimated by weighing a pipette, filling to the mark, draining, and again weighing to obtain the residual water in the pipette.

When using a 10 c.c. pipette for normal work, the error is not at all serious; but in the case of dilutions using a 1 c.c. pipette, it is advisable to rinse out the pipette by drawing up $\frac{1}{2}$ c.c. of water, and rolling this water round in the pipette to wash it out. The error introduced by the extra water added to 100 c.c. is much less than the possible error which would be present if the pipette were simply allowed to drain without rinsing. It is also advisable to use a fresh dry sterile pipette for each dilution, and to add one-twentieth to the results obtained.

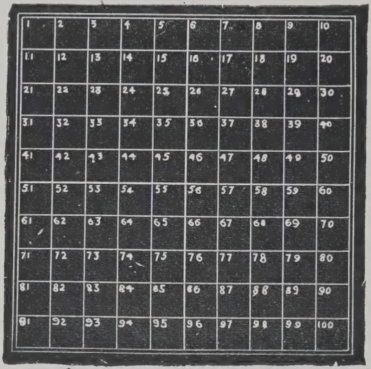
Some workers make a 10 per cent dilution by adding 1 c.c. of water to 9 c.c. of sterile water in a small flask; but for the reasons given, whenever practicable, it is better to work on the 10 c.c. pipette method. The greatest care should be exercised, because a very slight consistent error may eventually mean a final error of x 1,000,000.

Whichever method of dilution be employed, the next step is to make plate cultures from typical colonies grown at each dilution. Every type of colony is picked out and a streak or stab culture grown, from which cultures may be made in various media in order to ascertain the reactions of the organism.

Counting of colonies. When there is a small number only of colonies in a dish, these may readily be counted without the aid of any external appliance; but when a large number is present, unless some system is adopted to ensure the whole of the area being covered, it is highly probable the result will be erroneous.

One much used method consists in taking a piece of graph paper, inch squared, and by means of indian ink marking out half-inch squares alternately black and white, after the manner of a draught board. This is placed under the petri dish, and the colonies above each square are counted systematically.

A method which can be used when colonies are very numerous is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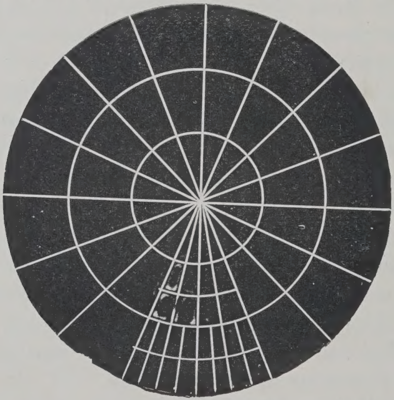
(*Baird and Tatlock*)

FIG. 167. COUNTING SHEET

to take a piece of cardboard the diameter of the top dish, and to cut out of this a sector, either a quarter, an eighth, or a sixteenth.



(*Baird and Tatlock*)

FIG. 168. PARKE'S COUNTING SECTOR

The mask thus made is placed on top of the dish and the visible colonies are counted. The disc is moved round into any position and again counted, the operation being repeated until at least a dozen counts have been made. These are averaged to find the average number of colonies per sector, and the number multiplied by 4, 8, or 16, as the case may be. Results may be stated by one of two methods—

(*A*) Bacteria present in 10 and 50 c.c. of original infected source. Bacteria absent in 1 and 0·1 c.c. of original infected source. Hence, concentration is between 100 and 1,000 per litre.

(*B*) Using the above figures, one bacterium is present in a volume of liquid between 1 and 10 c.c.

Anaerobic incubation. Certain bacteria are inhibited in their growth by the presence of oxygen to a greater or less extent. There are some types which grow best with such a small trace of oxygen that they are apparently completely anaerobic, but all, so far as is known at present, require at least a minute trace of oxygen.[1] These organisms are, therefore, divided for convenience into two rather vague classes: the obligative anaerobes, which will not grow under ordinary culture conditions, and require oxygen to be excluded to a considerable extent; and the facultative anaerobes, which will grow under ordinary atmospheric conditions, but thrive better when oxygen is partly excluded.

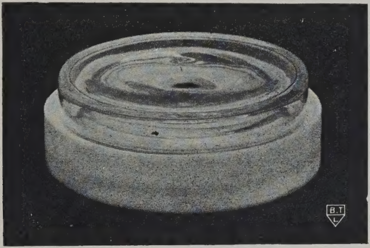


FIG. 169. McLEOD'S ANAEROBIC APPARATUS

The main methods for removal of oxygen are two in number—

1. EXHAUSTION OF OXYGEN. This may be done either by—

(*a*) The filter pump.

(*b*) Certain solutions, such as alkaline pyrogallol.

2. REPLACEMENT BY AN INERT GAS. The gas usually employed is coal gas, though now that hydrogen, nitrogen, and other inert gases are so easily obtainable compressed in cylinders, their use is preferable.

In practice, two of these methods are often combined; for example, the air may be removed largely by a vacuum pump, and the residual oxygen absorbed by alkaline pyrogallol; or the air may be replaced by hydrogen, an oxygen absorbent also being employed.

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[1] B. Tetanus has, however, been grown recently under extremely strict anaerobic conditions.

Filter Pump Method. A plate-glass base, or a flat ground-glass plate, is well greased with vaseline, and the petri dishes (or test tubes held in a small beaker) are placed on it. A bell jar is put over the apparatus, twisted round until the base makes an air-tight contact with the plate, and the air evacuated by means of a filter pump.

Absorption Method. 100 c.c. of a 10 per cent solution of KOH in water are taken, and 2·5 grm. of dry pyrogallol are dissolved in it.

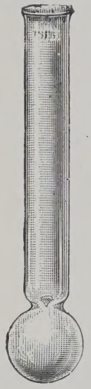

FIG. 170. BULB TUBE

A bell jar is employed, as in the previous method; but in place of the filter pump exhaustion a beaker or shallow dish containing the alkaline pyrogallol is placed under the bell jar with the other apparatus. The surface exposed should be as large as possible. This method is especially convenient when examining various cultures comparatively, but other methods may be employed which give better absorption. Creeping of the caustic potash may be minimized by means of paraffin wax.

(*a*) The test tube culture is placed inside a larger tube which is constricted at the bottom (see Fig. 170), and the bulb so formed is half-filled with the pyrogallol.

(*b*) The culture tube is fitted with a Buchanan ring, this being an annular ring fitting into the top of the test tube, containing the solution. This is rather awkward to handle, though some prefer it to the other methods.

(*c*) An *H* tube is employed, one limb of which contains the medium, the other the pyrogallol. A very convenient method if the tubes are in stock.

(*d*) In this method a tight cotton-wool plug is pushed down the test tube to be within an inch of the surface of the medium. A loose plug is then inserted to within half an inch of the first one, and on this is poured the pyrogallol solution. The cotton-wool plugs must be thoroughly sterilized, and the tubes incubated upside down.

(*e*) A very convenient method is to fuse a spot about 2 in. from the bottom of a test tube in the blow-pipe, and by means of the point of a lead pencil to push in the fused place. A small ignition tube containing the absorbent solution will rest against this protuberance.

(*f*) A further method is to insert a short glass rod about 2 in. long into the test tube before the medium is sterilized. This serves also as a rest for an ignition tube containing the pyrogallol.

Concentration technique. In the case of a liquid which is presumed to be infected by micro-organisms, a streak culture is usually made on agar, a stab culture on dextrose gelatine, and an agar spread plate. The agar should be incubated at 37° C. and the gelatine at room temperature, until evidence is obtained of growth. When no growth is visible after several days, it must not be assumed that there is no life in the liquid, because the organisms may be present in the infected source in such small numbers that none were included in the loopful which was used for the inoculation; they may, on the other hand, be plentiful, but of the anaerobic type which do not grow easily under aerobic conditions; or they may belong to the nitrifying or other classes of organisms which require special media for their ready growth. The fertility of growth on the agar slope is, however, some indication of the abundance of life in the culture.

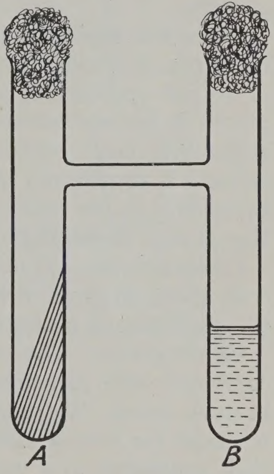

Fig. 171. H Tube for Anaerobic Work

The first case only will be considered at this juncture, i.e. that of a relatively sparse population in the liquid under examination. The first steps in the examination must evidently be to concentrate the organisms. This may be done in several ways, the most important being filtration, precipitation, evaporation under reduced pressure, and enrichment.

Enrichment. Of the above methods, enrichment is in general to be preferred, though in some cases it may give an erroneous impression, because of the suppression of one organism by the more rapid growth of another, or because the temperature of incubation or other conditions may not be quite appropriate for certain organisms. It is usually carried out by the addition of a fair quantity, say, 5–10 c.c. of the infected source to 10 c.c. of a double strength broth, and incubating for one to two days at 37° C. After this time the cultivation of a gelatine stab and an agar slope should usually give quite definite results.

The dangers are that some bacteria will multiply more rapidly than others, and hence there may be a smaller final percentage of some organisms than before, or even some organism may be swamped by the others. In the case where one species only is being sought, certain agents may be added which retard the growth of all bacteria, but retard the particular one wanted to a much less degree, thus, producing conditions favourable for its competitive growth. This may be illustrated by the use of sodium taurocholate in nutrient gelatine when seeking B. Coli communis. (See Water Analysis.)

Concentration by Mechanical Precipitation. Of the many methods which have been suggested, only one will be given, due to Fricker. It depends on the addition of ferrous sulphate to the liquid and the subsequent precipitation of ferrous hydroxide, which carries down with it a large proportion of the organisms present.

When this method is used in water analysis, 1 litre is taken, 4 c.c. of 10 per cent sodium carbonate is added, well stirred; and $3\frac{1}{2}$ c.c. of 10 per cent ferrous sulphate added with stirring. The water is placed in ice, and allowed to stand for several hours; or, in case a sufficiently large centrifuge is available, is centrifuged. The precipitate is transferred to a sterile flask or test tube, and dissolved by adding 25 per cent potassium tartrate with shaking. This solution is used to inoculate petri dishes in the usual manner.

Centrifuging. This is an excellent method, but when dealing with great dilutions a very powerful instrument is required. Continuous forms of centrifuge are now available.

Evaporation. The evaporation under reduced pressure is often combined with enrichment, and excellent results may be obtained in many cases. Nutrient broth is added to the liquid, and allowed to stand for two or three hours to prevent frothing. The flask is then connected to a vacuum pump and boiled at 37° C. until the required concentration is obtained.

When simple evaporation is attempted of a large volume of liquid, the apparatus shown in Fig. 172 should be used. The flask may be of any reasonable size.

Filtration. A sterile porcelain filter candle is employed in conjunction with a Buchner flask, filtering from within to without. The liquid to be filtered should be contained in a separating funnel, the neck of which is plugged with cotton wool to prevent inoculation from the air.

When filtration is complete, 10 c.c. of sterile water are added to the candle; if a volume of 1 litre has been filtered, the concentration is then *x* 100, and 1 c.c. of the concentrated liquid equals 100 c.c. of the original. This reckoning is sufficiently accurate for the average purpose.

Identification. Having brought about, by the plating methods outlined, the separation of the original mixture of organism into pure types, it now remains to sub-culture, for the sake of safety, at least three of each pure culture type, though only one need be worked out fully. The sub-culture process consists of again making plates with dilutions of 1 : 10 : 100, but at this stage the

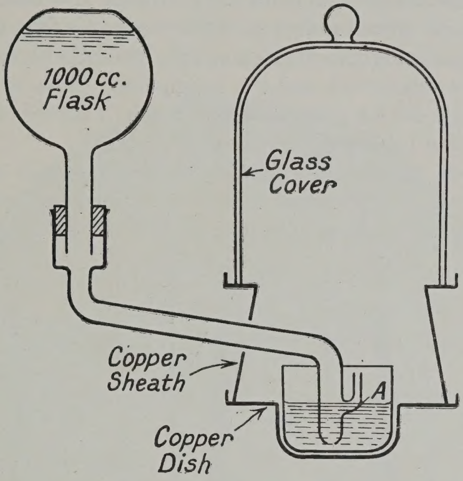
1000 cc.
Flask
Glass
Cover
Copper
Sheath
A
Copper
Dish

FIG. 172. APPARATUS FOR THE EVAPORATION OF A LARGE VOLUME OF LIQUID

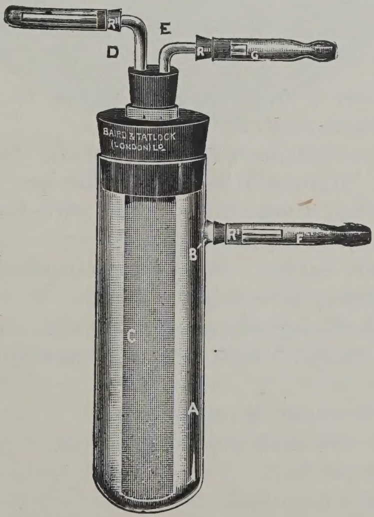
R'
E
D
R''
G
BAIRD & TATLOCK
(LONDON) Ld
R'
F
B
C
A

FIG. 173. FILTER CANDLE

information gained by observation of the preceding stages may be conveniently tabulated, and other experiments put in hand for the purpose of ascertaining the exact behaviour and reactions of the organism.

The complete routine method may now be summarized. A preliminary series of spread plates on nutrient agar is first made, for the purpose of separating the organisms into presumably pure colonies. The plates are examined under a magnifying glass or under a low power, and type colonies are selected. Each of these is then cultured according to the following routine—

(*a*) Plate out on nutrient gelatine, and make stab cultures from several colonies. The material should be stained by Gram's method. Dextrose gelatine stabs should also be made, and if gas is formed, fermentation tubes of dextrose broth should be prepared in order to identify the gases produced.

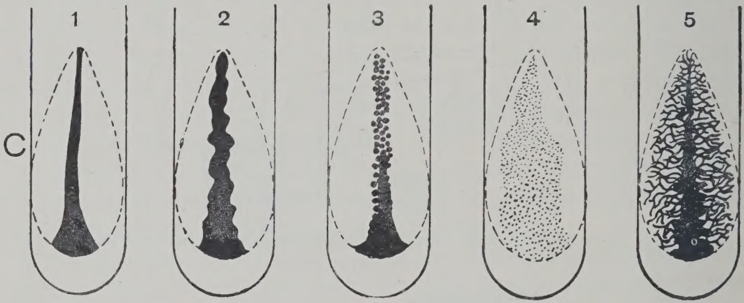

FIG. 174. TYPES OF STREAK CULTURES (AFTER FROST)
1 = Filiform. 2 = Echinulate. 3 = Beaded 4 = Effuse. 5 = Arborescent.

(*b*) Plate out on nutrient agar, and make streak cultures from several colonies. Stain with aqueous methylene blue.

(*c*) Make nutrient broth tubes; stain with Loeffler's methylene blue (No. 11) and test for Indol.

(*d*) Litmus milk culture. Stain for presence of envelope.

(*e*) Potato culture, for colour of growth.

The organisms thus grown should be examined according to the methods given below, which are conveniently divided into the following groups—

1. Behaviour towards culture media.
2. Appearance under high magnifications.
3. Staining properties.
4. End products of growth.

1. Behaviour Towards Culture Media.

(*a*) **Streak cultures.** SHAPE OF STREAK. The principal types are sketched in Fig. 174.

Impression Mount. The gelatine will slide out of the tube by placing it for an instant in hot water. A clean cover glass is lowered on to the surface, by means of a pair of cornet forceps, and tapped or pressed gently with the point of a small knife without allowing it to slide. It is then raised carefully by one edge, air-dried, and stained. The colony structure is then easily seen. The mount is of little value for identification.

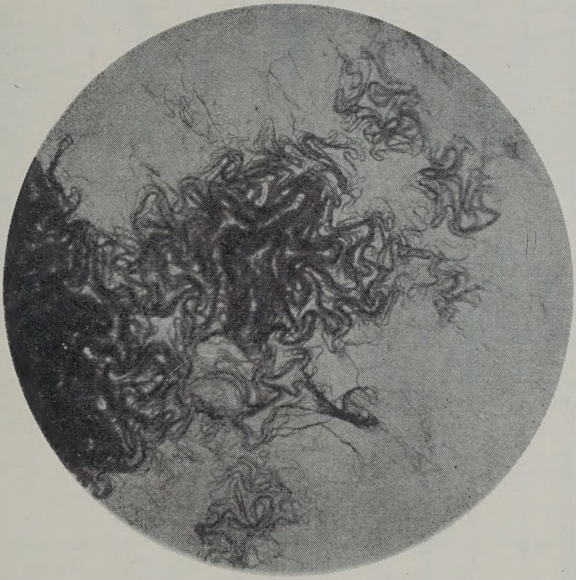

Fig. 175. Impression Mount from an Anthrax Culture

As for plate cultures for remainder of observation.

(*b*) **Stab cultures.** Growth along Track of Puncture. The chief effects are illustrated for both liquefying and non-liquefying organisms. Growth in the stab culture is a rough indication of the oxygen requirements of the organism. When growth occurs on the surface and also down the length of the track, the organism is aerobic and facultative anaerobic. Growth on the surface only, or to merely a short distance down the track, indicates large oxygen requirements; crateriform or stratiform lignefaction gives the same indication. Micro-aerophylic bacteria, to which oxygen, but only in small quantities, is necessary, grow at a distance of perhaps a centimetre deep from the surface.

Gas Production. Whether bubbles of gas are produced or not (especially with sugar media).

(*c*) **Broth cultures.** Reaction, Acid or Alkaline. This is usually tested by the addition of an indicator to the culture, enabling *pH* changes to be followed during growth; the reaction is sometimes at first acid, later becoming alkaline.

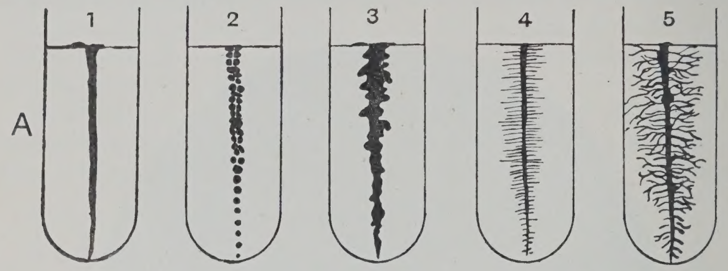

Fig. 176. Stab Culture Types, Non-liquefying Bacteria (after Frost)

1 = Filiform. 2 = Beaded. 3 = Echinate. 4 = Villous. 5 = Arborescent.

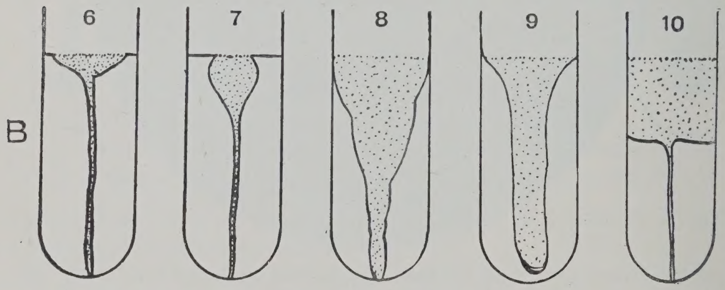

Fig. 177. Stab Culture Types, Liquefying Bacteria (after Frost)

6 = Crateriform. 7 = Napiform. 8 = Infundibuliform. 9 = Saccate. 10 = Stratiform.

Film Formation. Any scum or membrane forming on the surface should be examined carefully.

Sediment. Whether flocculent or dense, and its colour.

Coagulation. Whether coagulation occurs (with milk media), and whether the coagulant floats or sinks.

Smell. Many organisms produce a characteristic odour.

(*d*) **Plate cultures.** The colonies should be examined with the naked eye or with a magnifying glass (*e*, *f*, and *g* under low power also), noting the following points—

Speed of Growth and Temperature Range. Some organisms grow very slowly outside a particular temperature range, and by incubation of a suitable mixture at two temperatures, such as 10° C. and 37° C., the relative rates of growth of two organisms may be entirely reversed.

Direction of Growth. Whether down into the medium, under the surface, spreading on the surface, or above the surface, causing a small prominence. The vertical section along a diameter of the colony should be sketched.

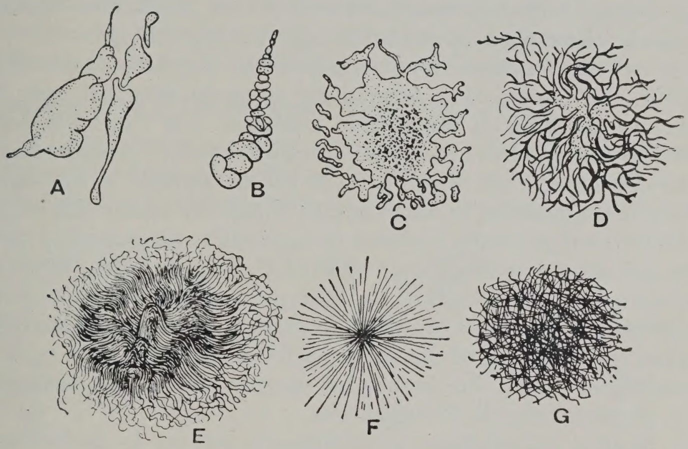

Fig. 178. Colony Types (after Frost)

A = Cochleate. *B* = Conglomerate. *C* = Amoeboid. *D* = Rhizoid. *E* = Curled. *F* = Mycelloid. *G* = Filamentous.

Size of Colony. The usual maximum and minimum sizes of colonies should be noted in millimetres diameter.

Shape of Colony. Whether a mere spot, oval, circular, or an irregular shape. The colony shape often varies greatly on different media.

Edge of Colony. Whether smooth, toothed, serrated, torn, hair-like, or comb-like.

Texture of Colony. Whether solid, watery, hard, fleshy, brittle, mass of threads (twisted or radiating).

Surface of Colony. Smooth, blistered, scaly, folded, or cracked.

Colour. Whether opaque, transparent; colour at edges and in middle.

Effect on Medium. Whether liquefied, coloured, or rendered fluorescent (e.g. when neutral red is added) acid or alkali production when indicator is added.

(*e*) **Sugar media.** The usual procedure is to incorporate a suitable sugar in nutrient gelatine, the most common being dextrose, lactose, and saccharose, other carbohydrates often employed being maltose, galactose, arabinose, xylose, mannite, and glycerine. Bacteria, or the enzymes which they produce, have always a selective action upon sugars; and in some groups, such as the large family of lactic acid bacteria, the varying action upon sugars is almost the only means of distinguishing between forms which are otherwise identical. Some organisms produce gas in sugar free gelatine.

The selective action of micro-organisms was first observed by Pasteur, who found that penicillium glaucum attacked only the dextro rotary form of ammonium racemate. On the other hand, various schizomycetes attack the laevo form only. Other examples of the selective action, which is by no means confined to the sugars, are the decomposition of laevo mandelic acid by aspergillus mucor, or dextro methyl propyl carbinol by aspergillus niger, and of propylene glycol by several organisms of the B. vulgaris type. When gas is produced (in a stab culture, for example), the fermentation should be carried out again in a Smith or Durham tube, the gases analysed, and the end products of the fermentation identified.

(*f*) **Litmus milk.** The action of bacteria upon this medium is often of great value in identification.

(*g*) **Neutral red gelatine.** In addition to serving as an indicator, this dyestuff on reduction causes fluorescence in the medium; a positive reaction is almost always of value.

(*h*) **Anaerobic cultures.** (Oxygen tolerance.) A general indication of the oxygen requirements may be obtained from the gelatine stab culture. Liquefying organisms, if aerobic, produce surface (crateriform or stratiform) liquefaction. Facultative anaerobic organisms, on the other hand, liquefy the whole of the track (infundibuliform or saccate). Non-liquefying organisms grow either the whole of the length of the track (facultative), or chiefly on the surface. Microaerophyllic organisms, which, though requiring oxygen, must only have this present in small quantities, grow about a centimetre from the surface of the stab.

(*i*) **Other media.** There are various special nutrient media, such as McConkey's lactose bile salt agar, which have been devised to retard the growth of all organisms except the group for which the test is to be made (in this case the B. Coli group).

Characteristics of colonies. In general, all tubes and plates should be examined after one, two, and three days, and at the end of a week. In some cases it is necessary to examine the culture after two weeks or more. The changes which occur as the culture grows older must be particularly noted; this applies more especially to observations under high powers, which will be dealt with below.

It should be remembered that, according to the temperature of incubation, the colour of the colonies may vary; normally sporing bacteria may not form spores; normally motile bacteria may be non-motile, and so on. A definite *pH* value of the medium may be necessary for the full development of the organism; or a special medium containing suitable chemicals; or air may require to be excluded. In some instances, therefore, growth may be slow or abnormal in character in the standard media employed.

2. Appearance of the Organism Under High Powers.

For this purpose, the petri dish must be opened just sufficiently to take a platinum wire. One of the colonies is scraped, and the extracted matter is stirred into a drop of sterile water or physiological salt solution on the microscope slide. A cover glass is put on and the mount examined. The lowest power which is serviceable is the 4 mm., but a dry $\frac{1}{8}$ in. objective is preferable. After some time the activity of aerobic organisms diminishes greatly, except at the edges of the cover glass, due to oxygen exhaustion.

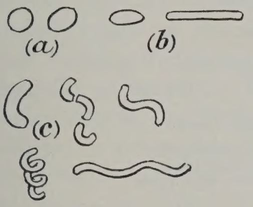

FIG. 179

a = Coccus. *c* = Spirillum.
b = Bacillus.

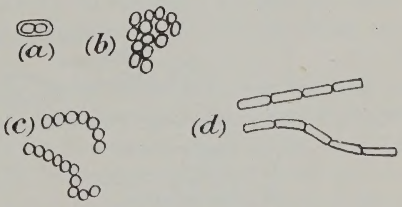

FIG. 180

a = Diplococcus. *c* = Streptococcus.
b = Zooglea or staphyl coccus. *d* = Chains of bacilli

The following points should be noted—

SIZE. The size of bacteria varies considerably, thus Micrococcus progrediens is only 0·15μ in diameter, whilst another coccus, Thiophyta volutans (a sulphur organism), may reach a diameter of 18μ. Similarly, bacilli range from Pseudomonas indigofera, which plays a part in the fermentation of indigo, length 0·2μ and breadth 0·06μ, to another sulphur bacterium, Beggiatoa mirabilis, which grows to 40μ wide and 20μ long.

THE SHAPE. The three main types are the bacillus, a straight cylindrical or rod-shaped organism, with more or less rounded ends; the coccus, a roughly globular or ovoid organism; and the spirillum, which may be considered to be a bacillus which is bent like a bow, or twisted like a spiral. (See classification of organisms, Chap. XIV.)

COHESION. Whether the organisms are separate; joined together, either end to end, side by side, or in groups of the types illustrated.

Motility. The very small bacteria have an apparent motion, which may be Brownian movement; true independent motion ceases if formaldehyde is introduced under the cover glass. The speed of movement may be measured by means of a stop watch and micrometer scaled eyepiece, in μ per second, unless the movement is very lively. Some organisms move so quickly that it is difficult to follow them with the eye. The speed of three common organisms is, roughly: B. megatherium, $7{\cdot}5\mu$ per second; B. subtilis, 10μ; and B. vulgaris, 15μ per second.

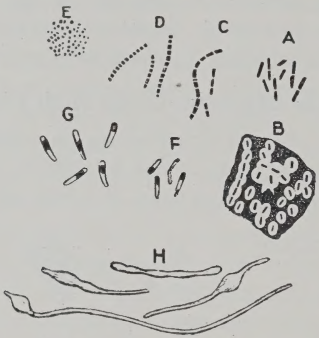

Fig. 181. Pseudomonas Syncyanea

A = Vegetative form.
B = Zooglea.
C = Chains of short rods.
D = Chains of cocci.
E = Coccus stage.
F and *G* = Sporing rods.
H = Involution forms.

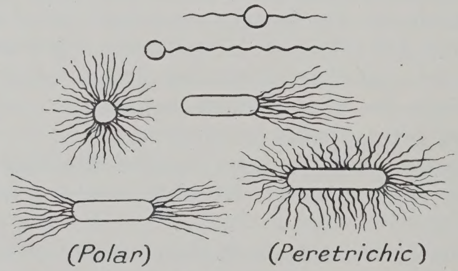

Fig. 182. Flagella Types

Involution Forms and Pleomorphism. These may be ascertained by patient observation, but are not normally very important in industrial work.

Flagella (or *cilia*). These hair-like appendages of micro-organisms are the most common cause of motility. They are difficult or impossible to observe without staining. The rod micro-organisms were in the older classification divided into three classes according to the absence or position of the flagella, the bacterium having none, the bacillus having them distributed over most of the body, and the pseudomonas having them at the ends or "poles" only. This classification is not now valid.

SPORES. Spore formation may occur in the centre of the organism or at one end. The staining technique for spores is given later.

CAPSULE. Many, if not all, bacteria have a membrane, which is occasionally visible without staining, as in certain sea-water organisms, but which usually requires staining to make it visible, and which in many instances has not yet been demonstrated.

INTERNAL CONTENTS. Oil globules, vacuoles, sulphur globules, and iron deposits may be present, in addition to certain reserve materials, such as volutin and glycogen. (These are dealt with under Staining Technique.)

Staining of bacteria. The main objects of staining bacteria are the elucidation of structure and the identification of the organism. Bacteria in general are normally colourless, and under ordinary conditions of observation display no evident internal structure; but they react very differently towards dyestuffs, and possess different staining properties according to the condition—alive, dead, or fixed. By far the greatest amount of staining is carried out on fixed bacteria, and for industrial purposes other methods, such as the so-called vital staining—which consists in staining the tissues of a living animal with, for example, neutral red—may be neglected.

As in the case of vegetable structures, many dyestuffs possess the power of selectively staining the internal structure of the organisms; whilst other dyestuffs, amongst which are methyl violet, safranine, capri blue, iodine green, thionine, and especially methylene blue, stain certain structures the colour of the dye, other structures taking on the shade of the colour base. Some bacteria stain entirely with the base of the dye. A dyestuff may be "polychromatized" by the addition of a little alkali, which enables the colour base to be more easily withdrawn from the stain, a very well-known example being Leishmann's stain, which consists of a partially polychromatized methylene blue, to which eosin is added; or Giemsha's stain, which is the same in principle, except that the polychromatized methylene blue product is isolated before mixing with the eosin. Methylene blue staining, and Gram's method are extremely important.

It is, as a rule, preferable to double stain in two processes; whilst in certain cases a mordant may be applied, such as tannic acid, before staining. Differentiation is also brought about after staining by the washing method employed, solutions of sulphuric or hydrochloric acid, chloroform, resorcinol or hydroquinone being employed according to circumstance, giving greater contrast.

In earlier years the majority of stains were of vegetable or mineral origin, but at the present time almost all the stains of value are synthetic dyestuffs, many of which owe their production entirely to the demand for them by microscopists. Many others are commercial dyestuffs, which are specially purified to meet the more exacting requirements of microscopic technique.

As a general rule, the special advantages gained by the use of these dyestuffs are increased by admixture with them of so-called "mordants," a vague term applied equally to such true mordants as tannin and such substances as aniline, which probably act largely as swelling and impregnating agents.

The most useful bacteriological stains (see pp. 362, 363) are—

Aqueous gentian violet.
Aniline water gentian violet (Ehrlich).
Loeffler's methylene blue.
Ziehl's carbol fuchsine
Aqueous Bismarck brown.
McCrorie night blue.
Gram's iodine.

The chief processes for staining bacteria are—

1. Dry staining.
2. Dry irrigation staining.
3. Wet irrigation staining.
4. Double staining.

DRY STAINING. This operation may be carried out either upon the slide or upon the cover glass; in general, the writer prefers the slide method. A drop of water is placed upon a perfectly clean slide, and by means of a platinum wire is infected with a little of a culture grown upon a solid medium (a better background is obtained than from a broth culture) until it is slightly milky. The drop is now spread evenly over part of the slide by means of the wire (or by making a sandwich of the drop between two slides and sliding them apart) and allowed to dry, preferably naturally. Judicious warming above a very small flame may be used, although there is danger of parts of the preparation being overheated. The slide, with the culture slide downwards, is then passed through a bunsen flame, in order to "fix" or kill the organism.

A large drop of the stain is now placed on the slide, or, when a cover glass is used, this may be floated upon a few cubic centimetres of the stain in a watch glass by gently lowering it on to the surface of the stain with a pair of pincers. It is allowed to remain in contact with the stain for a few minutes, depending upon the strength of the stain, the type of organism, and the experience of the operator; then drained, washed under a slow stream of water (a running tap will serve) for a second or so, and allowed to dry. A 1 per cent solution of almost any basic colour is suitable for use in this way.

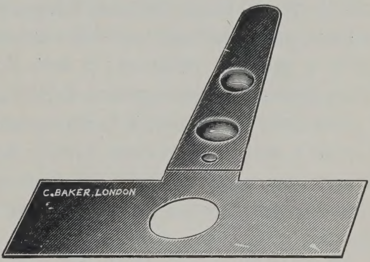

Fig. 183

The dry cover glass is then lowered gently on to a drop of Canada balsam and examined under the microscope. When a slide has been used, the mount may be covered with a drop of cedarwood oil and examined by means of any objective higher in power than one-sixth, using it as an immersion objective. In this way the use of a cover glass is avoided.

Dry Irrigation Staining. This method is used when there is available only a very small quantity of material, for the action of two or three stains may be observed on the same mount. This examination is also sometimes helpful in comparing the effect of several stains on one mount.

A mixture of vaseline and paraffin wax is made, and four tiny pellets are placed on a slide, sufficiently close together to act as the four supports to a cover glass, leaving a very small thickness of air between the slide and mount.

A quantity of the inoculated material is dried on to a cover glass, as described under dry staining, and fixed; the cover glass is then rested face downwards on the wax pellets. A large drop of water is now placed near one edge of the cover glass, and will quickly fill the space between that and the slide by capillary attraction. The bacteria are observed in this condition, and in particular the size of bacilli are noted.

By means of a piece of filter paper, the water is now almost sucked out from the opposite side to that at which it was introduced, and a drop of stain is then introduced under the cover glass in the same way. When the required observations have been made, the stain is washed out by irrigating with alcohol drops, and a second stain is then used on the same specimen.

Wet Irrigation Staining. This method is useful because bacteria can be stained in the living condition. A drop of the culture is placed on the slide, covered with a cover glass, and a drop of stain is placed touching the rim of the cover glass. The bacteria are observed which are at the junction of the stain and the mounting medium, any changes in condition being noted.

Double Staining. Under this heading a great many different types of process may be included, from those which treat with a dye, and subsequently apply a reagent which will remove it from certain types of matter, as in Gram staining, to those which successively stain with two complementary colours, e.g. red and green, this latter method being usually known as counterstaining. As so much depends on the conditions of strength, time, and other factors, which must be altered for various organisms, it is not possible to give a general method; a few representative examples are, therefore, given, which cover a fairly large field.

Gram's Staining of Bacteria. A very useful general test is Gram's staining process, which divides bacteria up into two classes, which are respectively stained at the end of the method, or are decolorised by the latter part of the method. Old cultures behave anomalously.

The specimen is dry stained on the cover glass with Ehrlich's stain No. 10 for 4 or 5 min., and the cover glass is then dipped in Gram's iodine solution No. 22, in a watch glass for 2 min. or rather less. The preparation is then washed in alcohol for about a minute and examined by being mounted in water; or, in the case of small bacteria, the cover glass is dried and mounted in Canada balsam.

Staining Flagella. In order to obtain good stains of the flagella, a certain amount of experience is required, for a stain which is too strong destroys them by swelling; and, on the other hand, unless they are stained fairly well, they are very difficult to see. These structures are very delicate, and any sudden changes of condition, either by rapid temperature change or rapid change in the concentration of liquids in which they are immersed, or quick transference from water into alcohol, for example, have a damaging effect. They may often be observed by mounting in indian ink.

The flagella are only present on motile bacteria, and then only during the motile period of the life history. All forms of bacteria exhibit flagella, but the distribution varies; in some cases they are found evenly all over the surface, as in many cocci; in other instances they may be grouped together at the end, or spread along the length of a bacillus.

A large drop of water is placed at each end of a slide, and one of these is infected from an agar culture by adding a small amount of the growth on the end of a platinum wire. After well stirring in, the second drop is inoculated from the first one by means of the amount of liquid which can be carried on the platinum wire. There are thus two concentrations of the organism.

Two perfectly clean cover glasses are now taken, and liquid from each of the two drops is spread evenly over the two cover glasses respectively; the preparations are then allowed to dry at the ordinary temperature.

They are then dry stained cold with night blue (16) and washed in cold water. After drying, they may be mounted in xylol and examined; but, unless the examination be carried out systematically by one of the methods described, the bacteria exhibiting flagella may be overlooked.

A better but more tedious method of staining flagella is described by J. Craigie, *Journal Royal Microscopical Society*, March, 1929; this relies upon the use of silver salts.

Staining capsules (envelope, sheath). The method is carried out by first mordanting with iron tannate, well washing with water, and staining hot with carbol fuchsine (14), also for 1 min. Many bacteria exhibit a skin which, by suitable methods, may be stained a different colour from the body of the bacteria; thus by the following method the bacteria are stained red, the capsules blue.

A little broth culture is picked out with a platinum wire and filmed on a cover glass. After drying, it is washed with water, and Muirs mordant applied for a few seconds only. Wash with water, methylated spirit and water, and counterstain with methylene blue for a short time. The washing with methylated spirits has a good deal to do with the success of the staining.

The water is removed with alcohol, the alcohol with xylol, and the mount made in Canada balsam. The envelope is thickest in older bacteria, and can often be seen in large bacteria without staining. The plasma of small bacteria may be shrunk by treatment with iodine-potassium iodide, when it becomes more evident.

Staining cell contents. The reserve materials found in bacteria are chiefly glycogen (starch), volutin (protein), and oil.

VOLUTIN. Volutin was first found by Mayer in Spirillum volutans as small round globules. It is colourless, strongly refractive, insoluble in organic solvents, very slowly soluble in cold water, and more soluble in hot water or in 5 per cent sulphuric acid. It may be detected by a wet irrigation stain with methylene blue, and gives a very pure blue tone after 5 min. In addition, it is stained by safranine and carbol fuchsine, but is left unstained by eosin, haematoxylin, and borax carmine. A dextrose agar slope culture is suitable.

Glycogen. This is tested for by means of iodine irrigation staining, examining after 5 min.; or a loopful of the culture may be mixed with a drop of the iodine solution, covered with a cover glass, and examined. It is easily dissolved out of bacteria by a short boiling in very dilute sulphuric acid or by the action of malt extract at 30° C.

Oil Globules. Under certain conditions oil globules may be mistaken for spores, but any doubt may be dispelled by mounting in chloral hydrate, when oil globules become invisible, whilst spores are as noticeable as before. A drop of culture is mixed with a drop of 40 per cent formaldehyde and, after standing for 5 min., a drop of methylene blue is added. The mixture is stirred with platinum wire for a few minutes, and after a quarter of an hour a little Sudan III stain (19) is added on a platinum wire. A little of the mixture is transferred to another slide and examined under a cover glass. The fat globules are red and the membrane pale pink. Blue-staining indicates cytoplasm. An agar slope culture incubated for 36 hours at 30° C. is usually successful.

Staining of spores. Spores vary in size and shape in various bacteria; often they are floating free, but they also may appear rather like oil globules inside the bacteria, either in the middle or near the ends of bacilli. They may be shown to have two walls, the inner one very thin and difficult to observe, the outer one easily seen.

One of the most successful methods depends on double staining with methylene blue and carbol fuchsine. It may be carried out in a few ways, of which two are given.

In each case, place a drop of water on the cover glass, and infect until slightly milky. Spread out by means of a platinum wire and allow to dry; then fix in the usual way by means of a flame.

Method A. 1. Gram's Iodine. Stain from 1 to 3 min. and wash with alcohol, then with water.

2. Carbol Fuchsine (46). Float on the hot stain for 1 min., dip for an instant into 20 per cent sulphuric acid, and put into water.

3. Methylene Blue (34). Put a large drop of methylene blue on to the cover glass for a few seconds, wash with water, and examine with a high power.

Method B. 1. Carbol Fuchsine (14). Stain as above, wash with water, dip for a few seconds into 3 per cent hydrochloric acid, and wash with water.

2. Loeffler's Methylene Blue (11). Stain for 3 min., wash with water, dry, and mount in Canada balsam.

Special end products. The tendency to-day is to ascertain the reactions which an organism can carry out, and rely on these rather than on morphology for identification purposes.

FERMENTATION TESTS. During the preliminary investigation, it will have been observed if gas is produced by the action of the organism on dextrose gelatine. If gelatine is not liquefied, this medium should be used, otherwise agar is better. Should gas be noted, a Smith or a Durham tube is inoculated, the latter being more useful for a qualitative examination, the Smith tube being preferable in general, as it allows the gas to be examined, estimated, and the organisms to be grown under more controlled conditions. The medium is sugar broth. A tube is inoculated, incubated at 30° C. for some time, after which a few cubic centimetres of 10 per cent NaOH are added to the short tube. The thumb is placed on the open end and the tube is slowly inverted a few times, the gas being kept in the long tube. Any diminution of volume indicates CO_2; it should be remembered that water dissolves its own volume of this gas. The remaining gas may now be changed over into the shorter tube and a match applied. If the gas burns or causes a slight explosion, methane or hydrogen is indicated. The broth liquid may be examined for the presence of alcohol lactic or other acids, or any definite end products which may be thought probable.

ACIDITY CHANGE. In comparative work this is often of value. A definite amount of the medium, say 50 c.c., is placed in a conical flask, inoculated by a definite amount of the source, e.g. 1 c.c., and incubated for three days. The liquid is then titrated with N/10 NaOH, a blank experiment being titrated as a check; it is of the greatest importance, if results are to be repeated or to become comparative, for the experiments to be carried out under carefully controlled and recorded conditions.

Approximate records of the acidity changes may be noted by making use of the Universal Indicator of British Drug Houses; the initial, final, and intermediate *pH* degrees should be taken.

INDOL. This is probably the most readily detected end product of protein decomposition by bacteria, and two tests are in general vogue for its recognition in a broth culture. Two or three drops of a 1 per cent $NaNO_2$ solution are added to the tube and, after shaking, a little concentrated HCl is run slowly down the side of the test tube, which is held in an inclined position. The test tube is brought carefully into the vertical position, when a red ring will be seen, due to the nitroso derivative of indol. Occasionally indol does not immediately react, in which case incubation may be carried out a little longer. Sulphuric acid alone will produce a pink ring when indol is present in quantity.

A second and more certain method of testing for indol depends upon its reaction with p. dimethylamino benzaldehyde hydrochloride in alcoholic solution, in the presence of excess acid and an oxidizing agent. The reagent is—

0·25 grm. p. dimethylaminobenzaldehyde;
10 c.c. concentrated HCl;
100 c.c. absolute alcohol.

One cubic centimetre is added to 1 c.c. of the broth, and about 1 c.c. of saturated potassium persulphate is run in, producing a pink colour if indol is present.

Sulphuretted Hydrogen. A broth without sugar is used for this test, in an ordinary test tube, a strip of paper which has been soaked in basic lead acetate being inserted in the tube and held in place by the cotton-wool plug. As a confirmation, an agar tube may be melted and a little lead carbonate (made into a cream with a drop of sterile water) added. The tube is allowed to set, and a streak culture made. The growth will, of course, be black if H_2S be produced.

Test for Nitrites. Infect a tube of peptone water, incubate at 28° C. for 1 week, and add 1 c.c. of—

(*a*) 0·5 grm. sulphanilic acid;
150 c.c. acetic acid, s.g. 1·04;

or (*b*) 0·1 grm. alpha naphthylamine acetate;
20 c.c. water; filter through cotton wool, cool, and mix filtrate with 180 c.c. dilute acetic acid.

If nitrites are present, a pink coloration is produced, due to the coupling of the diazotized bases.

Test for Ammonia. Incubate in broth for 48 hr. in a tube which contains in the neck a piece of Nessler's reagent paper.

Test for Enzymes. Incubate a stab culture for several days until all the gelatine is lignified. Add 1 drop of 5 per cent phenol per cubic centimetre of medium, shake well, filter, pour into sterile gelatin and into milk, and note the change which occurs.

Coloured End Products. Glycerine potato is the medium employed; it is an excellent means of growing chromogenic bacteria. A slab of potato about ½ in. diameter and ¼ in. thick is soaked in 0·1 per cent sodium carbonate for a day, then in 5 per cent glycerine for another day. A small lump of plasticine is now placed in the bottom of a test tube and the potato pressed down on to it by means of a glass rod. A thin layer of water should have been previously run in, not deep enough to cover the plasticine. The tube is plugged as usual.

All colours of the spectrum are produced by bacteria, some examples being: red, *B. prodigiosus*; orange, *micrococcus glutinis*; yellow, *sarcina aurantiaca*; brown, *B. brunneum*; violet, *B. violaceus*; black, *microspirans nigricans*. Beijerinck divides chromogenic bacteria into two classes—

Chromophoric (e.g. the purpur bacteria), in which the pigment is essential to life and is found in the protoplasm.

Chromopharic (e.g. all other bacteria), in which the pigment is a by-product of the vital chemistry, and is found outside the cell. There are also the parachromic organisms of this class, which exhibit the pigment partly in and partly out of the cell. Some pigments are soluble in water, others in certain organic solvents, whilst a few are soluble only in an alkali.

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CHAPTER XIV

BACTERIA

DURING the last half century, rapid and often sweeping changes have been made in the classification and nomenclature of bacteria. In consequence, one and the same organism may have had many names; for example, Bergey gives twenty-five names for *Clostridium butyricum* Prazmowski. To add to the confusion, the same generic name has often been given very different meanings by different emendators; thus, bacillus in the system of Migula denotes a rod-shaped organism having flagella, whilst endospores may or may not be formed; the name in the Society of American Bacteriologists (S.A.B.) classification, however, denotes a spore-forming rod, which may or may not have flagella.

The system of Migula formed the basis of most classifications proposed since 1898, and, on the whole, the nomenclature conforms to this in the literature. However, although it had the great merit of simplicity, it was found in many instances to be at fault in the grouping of organisms, related forms being placed in diverse groups, and several attempts have been made to amend it. The most important of these is to be found in Bergey's *Manual of Determinative Bacteriology*, and is due to a committee of the Society of American Bacteriologists. The present chapter is based upon this scheme, and the descriptions of organisms are taken from the third edition of that manual.[1] Many very familiar names have been altered, and it comes as something of a shock to find *B. coli communis* renamed as *Escherichia coli*, but the new nomenclature is gradually creeping into the literature. The classification cannot be said as yet to be entirely satisfactory, but it is probable that no serious change in principle will occur in future emendations.

A most comprehensive account of the various systems is found in Bergey's introduction; only one of these, in addition to the S.A.B. scheme, is outlined below. This is a slight modification of Migula's system given by Fuhrmann (*Vorlesungen über technische Mykologie*).

First order. COCCACEAE (Zopf.) emend. Migula. Cells spherical in the free condition, before division usually becoming somewhat elliptical.

(*a*). Non-motile. 1. *Streptococcus,* Billroth. Division in one plane only. In chains made up of a few to several hundred cells. Never in clusters. Cocci or single chains may have an envelope.

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[1] By kind permission of the Williams & Wilkins Company.

2. *Micrococcus* (Hallier), Cohn. Division in two planes.

3. *Sarcina,* Goodsir. Division in three planes.

(*b*) Motile. 1. *Planococcus,* Migula. Division in two planes.

2. *Planosarcina,* Migula. Division in three planes.

BACTERIACEAE, Migula. Cells cylindrical, short or long, dividing in one plane only, at right angles to the long axis, and elongating to twice the normal length before division. No sheath or envelope enclosing chains of individuals. May or may not form endospores.

(*a*) Non-motile. 1. *Bacterium,* Ehrenb. Cells straight, rodlike, no sheath, no flagella.

(*b*) Motile. 1. *Bacillus,* Cohn. Cells have flagella distributed over any part of the body. Endospores often formed.

2. *Pseudomonas,* Migula. Flagella formed at poles only, and rare endospore formation.

SPIRILLACEAE, Migula. Cells spiral or bent, division at right angles to long axis, no envelope.

(*a*) Non-motile. 1. *Spirosoma,* Migula. No flagella.

(*b*) Motile. 1. *Microspira,* Schroeter. Usually one, more rarely two or three undulating flagella.

2. *Spirillum,* Ehrenb. Polar flagella tufts, very rare endospore formation.

3. *Spirochaeta,* Ehrenb. Cells are themselves sinuous.

CHLAMYDOBACTERIACEAE, Migula. No sulphur content. Cylindrical cells united to form threads which are enclosed by a more or less thick envelope. During propagation, conidii, which may be either motile or non-motile, are produced. The cells change to this form either with or without intermediate and special division forms. The conidii change by division into new cell threads without an intermediate resting stage.

(*a*) Threads unbranched. Division at right angles to axis only. 1. *Chlamydothrix,* Migula. Non-motile cylindrical cells, both ends of thread the same appearance, thin divisions, division in one plane only, iron often present.

(*b*) Threads unbranched, but division previous to forming conidii occurs in three planes.

1. *Crenothrix,* Cohn. Envelope clearly visible. Usually contain much iron. Vegetative forms divide at right angles to long axis only.

2. *Pragmithidiothrix,* Engler. Envelope hardly visible. The cells forming conidii, initially bound to one unbranched thread, divided in three directions in space.

(*c*) Threads branched. 1. *Cladothrix*, Cohn. Cylindrical cells, which branch by longitudinal division. Propagation occurs by formation of clusters of conidii at the end of the vegetative cells. Iron not found in quantity.

Second order. RHODOBACTERIA. Distinguished by the presence of bacteriopurpurin and bacteriochlorin pigments, red to violet in colour. The morphological form may be globular, rod-like, or spiral.

1. *Thiorhodaceae.* Cells contain free sulphur.

Sub-families:
Thiocapsoceae
Lamprocystaceae
Thiopediaceae
Amoebobacteriaceae
Chromatiaceae
Rhodocapsoceae

2. *Athiorhodaceae.* No free sulphur content in protoplasm.

Sub-family: Rhodocystaceae.

Third order. THIO BACTERIA: the cells contain free sulphur in large or small globules, but are colourless.

BEGGIATOACEAE. Cylindrical cells, united in threads, no envelope surrounding chains, motility due to an undulating membrane.

1. *Thiotrix*, Winogradsky. Unbranched, fine sheath, non-motile, division at right angles to axis only, but conidii formed at ends of threads.

2. *Beggiatoa*, Trevisan. No envelope, no conidii observed, motile by undulation of thread.

THIOBACTERIACEAE. Globular, rods, or spirals; no threads formed.

The S.A.B. classification.

The following scheme is taken from the third edition of Bergey's *Manual*—

Order 1—Eubacteriales. Spheres and rods, containing no visible particles of sulphur or iron compounds. Motility, endospores, pigment, and reserve materials (volutin, glycogen, fat) may be present or absent.

Order 2—Actinomycetales. Spheres, short or long rods, spirals, filaments, the longer forms showing branching; pigment, bacteriopurpurin, bacteriochlorin; sulphur granules may be present or absent. Endospores are absent, but conidia may be formed by segmentation of the tips of filaments.

Order 3—Chlamydobacteriales. Algae-like filaments, often showing false branching. Sheath is present, often containing iron oxide. Conidia may be formed, but no sulphur or bacteriopurpurin is contained.

Order 4—Thiobacteriales. Spheres, short or long rods, spirals, filaments. Spore formation is rare; conidia are not formed. There is no sheath. Sulphur granules, bacteriopurpurin, and bacteriochlorin are present.

Order 5—Myxobacteriales. Motile rods, which secrete a slime. Endospores, conidia, sulphur granules, bacteriopurpurin, and bacteriochlorin are absent.

Order 6—Spirochaetales. Protozoa like slender flexible spirals. Endospores are not formed. Multiplication is by both transverse and longitudinal division.

Order 1 (*Eubacteriales*) is considered in some detail in the present chapter, the remaining orders being represented only by the more common species. The tables give a bird's-eye view of the properties of the more important bacteria in the several families of the first order. These particulars are entirely insufficient in themselves definitely to identify an organism, but they point to a probable organism, the more detailed description of which will be found in following the table. Bergey alone gives several hundred bacteria, and there are certainly many times that number either incompletely described or as yet unknown; it is, therefore, impossible in these pages to give more than a few of the more commonly occurring organisms. The field of industrial bacteriology is in many directions very incompletely explored, and it is certain that, in any comprehensive work carried out, new organisms will be encountered. In this case, after the characteristics have been ascertained, a stab culture should be made, and the tube sent to the National Collection at the Lister Institute. (From this Institute pure cultures of most of the important bacteria may be obtained.)

Order 1—Eubacteriales. This order is subdivided into five families—

Family 1. Nitrobacteriaceae.
Family 2. Coccaceae.
Family 3. Spirillaceae.
Family 4. Bacteriaceae.
Family 5. Bacillaceae.

1. **Nitrobacteriaceae.** Rods, and occasionally spheres, endospores rare; life energy derived from S, C, H, N, or simple compounds of these elements.

Tribe 1. *Nitrobactereae.* Oxidize simple compounds of C or N.

Genus 1. Hydrogenomonas: can live in absence of organic matter by the oxidation of hydrogen to water.

Genus 2. Methanomonas: short rods, which can grow in the absence of organic matter, by the oxidation of methane to CO_2 and water.

Genus 3. Carboxydomonas: rods, which oxidize CO to CO_2.

Genus 4. Nitrosomonas: Rods, with polar flagella, which oxidize ammonia to nitric acid, and can grow in the absence of organic matter.

Genus 5. Nitrosococcus: large spheres, which do not grow on ordinary media, but oxidize ammonia to nitrous acid in the soil and suitable media

Genus 6. Nitrobacter: non-motile rods, not growing well on ordinary culture media; oxidize nitrous acid to nitric acid.

Genus 7. Acetobacter: rods, chains, often motile, obligate anaerobes, showing involution forms, which oxidize alcohol to acetic acid.

Genus 8. Thiobacillus: small rods, which oxidize sulphides or sulphur to sulphates, and can derive carbon from CO_2 or carbonates in solution.

Tribe 2. *Azotobacterieae.* Genus 9. Azotobacter: large rods or cocci, which oxidize carbohydrates, and can fix atmospheric N if combined N is deficient. Often motile with polar flagella; obligate aerobes.

Genus 10. Rhizobium: small rods, showing branching forms, motile when young, obligate aerobes; can fix atmospheric N if combined N is deficient; form nodules on the roots of leguminous plants.

2. **Coccaceae.** Cells are spherical, though elliptical before division. Occasionally motile, usually no endospores. Occur singly, in pairs, tetrads, packets, chains, and swarms. Often form pigments.

Tribe 1. *Streptococceae.* Division in one plane. Pairs and short chains, never packets. Gram positive. Grow well anaerobic, but not well on serum-free media. Pigment is white or orange.

Genus 1. Diplococcus: occur in pairs, encapsulated. Hexoses often fermented to acids. Parasitic.

Genus 2. Streptococcus: occur in chains or pairs, with rare capsules, and rarely swarm. Stab cultures show little surface growth and gelatine is rarely liquefied. Nitrates are rarely reduced. Usually parasitic.

Genus 3. Leuconostoc: pairs, chains, and swarms. Occasionally Gram negative. Occur in cane-sugar solutions. Saprophytes.

Tribe 2. *Neisserieae.* Division in one or two planes, occur in pairs and tetrads, and singly. Grow well only on serum media.

Genus 4. Neisseria: Gram negative, grow best at 37° C.

Genus 5. Gaffkya: Gram positive, occur as tetrads in the animal body.

Tribe 3. *Micrococceae.* Occur singly, in pairs, and in swarms. Usually Gram positive.

Genus 6. Staphylococcus: division in one or two planes, moderate growth on ordinary media, gelatine usually liquefied, simple carbohydrates fermented to acid, nitrates often reduced, any pigment white or orange, usually Gram positive. Grow well at 37° C., usually parasitic.

Genus 7. Micrococcus: occur in plates and swarms, never as packets or chains. Dextrose usually fermented to acid, lactose unchanged, gelatine often liquefied slowly, grow well on agar, pigment rare and yellow orange. Saprophitic or facultative parasitic.

Genus 8. Sarcina: division in three planes, forming packets. Usually Gram positive. Dextrose usually fermented to acid, lactose usually not attacked, gelatine often liquefied, grow well on agar; any pigment is yellow or orange, nitrates often reduced. Saprophytic or facultative parasitic.

Genus 9. Rhodococcus: occur in swarms, dextrose fermented to acid, lactose not attacked, gelatine usually liquefied, pigment is red, usually Gram positive; nitrates reduced to nitrites, but not to ammonia. Saprophytic.

3. **Spirillaceae.** Spiral or curved rods, not flexuous; no endospores, often motile (polar flagella) division always transverse, grow well on ordinary culture media, water forms or intestinal parasites.

Genus 1. Vibrio: short bent rods, rigid, single or in spiral chains. Usually one polar flagella, gelatine usually liquefied, usually aerobic, no endospores, Gram negative, active ammonifers.

Genus 2. Spirillum: rigid long curved rods or parts of a spiral; length, pitch, and thickness variable. Polar tuft of from 5–20 flagella, at one or both ends, occurring in water and putrid effusions.

4. **Bacteriaceae.** Straight rods, often motile, no spores formed, bacteriopurpurin, bacteriochlorin, pigment, sulphur granules not formed. Simple compounds of C, H, N, S cannot be utilized as food. Carbohydrates usually fermented to acid, gas also often formed. Starch may be hydrolyzed.

Tribe 1. *Chromobacterieae.* Saprophytic or parasitic for plants. Grow best below 30° C., Gram negative, pigment red, yellow, violet, or blue-green. Grow well on culture media. Occur in water and soil.

Genus 1. Serratia: small aerobic rods, Gram negative, flagella peretrichic; form pigment "prodigiosin" as red or pink. Gelatine rapidly liquefied, nitrates reduced, milk coagulated and digested, hexoses fermented to aliphatic acids, acetyl methyl carbinol, and 2.3. butylene glycol.

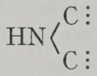
Genus 2. Flavobacterium: small aerobic rods, often motile, usually Gram negative, flagella polar or peretrichic. Pigment on gelatine or agar is yellow. Hexoses fermented to acid, but no gas.

Genus 3. Chromobacterium: small aerobic rods, forming on solid media violet pigment soluble in alcohol, insoluble in chloroform.

Genus 4. Pseudomonas: small aerobic rods, often motile, usually

Gram negative, forming green-blue pigment soluble in water, which diffuses into the medium. Water and soil organisms.

Tribe 2. *Protaminobactereae.* Gram negative, often pigment formed (red or yellow); grow not too well on culture media, attack lower alkylamines. Water and soil bacteria.

Genus 5. Protaminobacter: Non-motile, attack substances containing the group  $HN\left\langle\begin{matrix}C:\\C:\end{matrix}\right.$

Tribe 3. *Cellulomonadeae.* Often motile, often form pale yellow pigment, Gram negative, grow moderately on culture media, digest cellulose. Soil bacteria.

Genus 6. Cellulomonas: small rods, rounded ends, no spores, occurring in the soil.

Tribe 4. *Achromobacterieae.* Small to medium-sized rods, no pigment on gelatine or agar, brown on potato, variable action on hexoses, often motile, Gram negative.

Genus 7. Achromobacter: characters as tribe.

Tribe 5. *Erwineae.* Plant pathogens, and may kill the host. Cause blight and similar diseases.

Genus 8. Erwinea: motile rods, with peretrichic flagella, rods white, but rare pigment produced.

Genus 9. Phytomonas: motile, yellow or white rods, often produce yellow pigment.

Tribe 6. *Lactobacilleae.* Non-motile, gram positive rods. Carbohydrate fermented to lactic acid, any gas being CO_2 without H. Somewhat thermophylic, often microaerophylic, poor surface growths.

Genus 10. Lactobacillus: as tribe.

Tribe 7. *Propionbacterieae.* Non-motile, gram positive, long irregular rods (aerobic growth), short rods (anaerobic). Ferment carbohydrates, lactic acid, and polyalcohols, forming propionic and acetic acids, with CO_2. Complex N compounds required for growth. Catalase enzyme formed; often pigment also.

Genus 11. Propionibacter: as tribe.

Tribe 8. *Kurthieae.* Gram positive, free growth on artificial media, carbohydrates not attacked.

Genus 12: long rods, forming evenly curved chains, gram positive, motile, proteus-like growth on media. Carbohydrates not attacked, sulphuretted hydrogen not formed, occur in decomposing materials.

Tribe 9. *Pasteurelleae.* Gram negative, bipolar staining, non-motile, parasitic, fermenting powers weak.

Genus 13: facultative aerobic, no gas in fermentation, gelatine not liquefied, parasitic, frequently pathogenic to animals.

Tribe 10. *Klebsielleae.* Short plump rods, rounded ends, capsule, non-motile, gram negative, ferment carbohydrates to acid and gas, aerobic, grow well on culture media, found in the respiratory tract on man.

Genus 14. Klebsiella: as tribe.

Tribe 11. *Hemophileae.* Minute parasitic organisms, grow well only in special media, e.g. hemoglobin, non-motile, gram negative.

Genus 15. Hemophilis: as tribe, form threads, pleomorphic.

Tribe 12. *Bacteriaceae.* Gram negative, grow well on culture media, carbohydrates often fermented to both acid and gas, some liquefy gelatine, flagella usually peretrichic.

Genus 17. Escherichia: often motile, carbohydrates fermented to acid and gas, no acetyl methyl carbinol produced, often occur in the intestinal canal of normal animals.

Genus 18. Aerobacter: often motile, produce acetyl methyl carbinol. Often occur in the intestinal canal of normal animals.

Genus 19. Proteus: rods, highly pleomorphic, filaments, Gram negative, motile, flagella peretrichic, characteristic amoeboid colonies in most media, decompose proteins, ferment dextrose and usually sucrose, but not lactose, no acetyl methyl carbinol produced. Occur in putrefying materials.

Genus 20. Salmonella: often motile, occur in inflamed intestinal canal of animals.

Genus 21. Eberthella: motile, occur as 20.

Genus 22. Shigella: non-motile, occur in intestinal canal of man in dysenteric states.

Genus 23. Alcaligenese: often motile, occur in the intestinal canal of normal animals.

Tribe 13. *Bacteroideae.* Good growth on ordinary media, obligate anaerobes.

Genus 24. Bacteroides: as tribe; infect intestinal canal of normal animals.

• BACILLACEAE. Endospores formed. Gram positive rods: flagella peretrichic, if any; protein media decomposed by enzymes formed.

Genus 1. Bacillus: aerobic, saphrophytic, rods, often long chains, form of rod not greatly changed at sporulation, gelatine liquefied usually, gelatine colonies amoeboid in shape, Gram positive when young.

Genus 2. Clostridium: anaerobic or microphylic, often parasitic, rods often enlarge at sporulation.

TABLE V

Tabular Key to Coccaceae

Pigment : Gel. or Agar	Pigment : Potato	Stab Liquefaction	Milk : Coagulation	Milk : Peptonization	Milk : Litmus	Sugar : Dextrose	Sugar : Lactose	Sugar : Sucrose	Broth	Oxygen	Indol	Nitrates	Gram	Opt. Temp.	H_2S	*Coccaceae*
–	–	–	+	–	*R*	*A*	*A*	(*A*)		±	–	–	+	?		Streptococcus lactus
–		–								+				25		Leuconostoc mesenteroides
–	–	–			*R*	*A*	*A*			±	–	–	+	37	–	Gaffkya tetragena
–	*Y*	–	–		*R*	*A*	–	*A*		+	–	–	+	25	–	Micrococcus aurantiacus
–	*Y*	–	–		*R*	–	–	–		+	–	–	+	25	–	Micrococcus candicans
Y	*Y*	+	+		*B*	–	–	–		+	±	*Rd.*	+	25	+	Sarcina lutea
O	*Y*	+	+	+						+	±	–	+	30	–	Sarcina aurantiaca

Abbreviations: *R* = red, *O* = orange, *Y* = yellow, *G* = green, *B* = blue, *Gr.* = grey, *Br* = brown, *P* = polar, *T* = terminal, *I* = infundibuliform, *S* = saccate, *C* = crateriform, *H* = horizontal, *A* = acid, *G* = gas, *Rd.* = reduced.

Family 2. Coccaceae.

Streptococcus lactis (Lister), Lohnis; formerly *Bacterium leichmanni,* Wolff. Spheres, with many cells slightly longer than the breadth; dimensions usually fall between 0·5 to 1 micron; pairs and short chains, with an occasional culture showing long chains. *Whey gelatin stab:* filiform to beaded growth, medium in amount; no liquefaction. *Agar plates:* small, round or oval, grey entire edge, only slightly raised. *Agar streak:* tendency to formation of small definite streaks throughout growth area; in some portions growth becomes confluent. *Dextrose broth:* usually, first, a turbidity and, later, a sediment. Young cultures entirely reduced except for a narrow red band at top; the red band increased in width as the culture ages. No gas. No evident digestion, although whey may be expressed. *Potato:* no visible growth.

Leuconostoc mesenterioides. Spherical, 0·9 to 1·2 microns in diameter, occurring in pairs and in short or long chains. In sugar solutions the chains are surrounded by a thick, gelatinous, colourless membrane, consisting of dextran. *Dextrose gelatin colonies:* small, white to greyish-white, raised, nodular. *Dextrose gelatin stab:* growth along entire stab; no liquefaction. *Dextrose broth:* abundant growth, with massive formation of slimy material. *Occurrence:* in juice of sugar-cane and beets in sugar factories.

Gaffkya tetragena. Formerly *Micrococcus tetragenus,* Gaffky. Spheres: 0·6 to 0·8 micron in size, with pseudocapsule (in body fluids) surrounding four of the elements showing typical tetrads. *Gelatin colonies:* small, 0·8 to 2 mm. in diameter; white, convex. *Gelatin stab:* thick, white surface growth; no liquefaction. *Agar colonies:* circular, white, smooth, glistening, entire. *Agar slant:* white, moist, glistening. *Broth:* clear, with grey viscous sediment. *Potato:* white, viscid.

Micrococcus aurantiacus (Schröter), Cohn; formerly *Bacterium aurantiacum,* Schröeter. Spheres: slightly oval, 1·3 to 1·5 microns, occurring singly and in small clumps. *Gelatin plate:* circular to oval, smooth, glistening with yellow to orange centre. *Gelatin stab:* yellow surface growth; no liquefaction. *Agar plate:* circular, smooth, glistening, yellow to orange, entire. *Agar streak:* buff to orange-yellow, beaded growth, raised, glistening. *Broth:* turbid, with pellicle. *Potato:* slimy, yellow growth; pigment is insoluble in alcohol and ether.

Micrococcus candicans Flügge. Spheres: 1·0 to 1·2 microns, occurring singly and in irregular clumps. *Gelatin plate:* circular, porcelain white, glistening, slightly raised. *Gelatin stab:* white, glistening, raised surface growth; no liquefaction. *Agar plate:*

circular, white, smooth, glistening, contoured, entire. *Agar streak:* thick, white, raised, glistening. *Broth:* turbid, with pellicle, becoming clear. *Potato:* scant, thin, yellowish-white streak.

Sarcina lutea Schröter. Spheres: 1·9 to 1·5 microns, showing packets in all media. *Gelatin plate:* circular up to 5 mm. in diameter, sulphur-yellow, sinking into the medium. *Gelatin stab:* slow infundibuliform liquefaction. *Agar plate:* yellow, coarsely granular, circular, raised, moist, glistening; entire margin. *Agar streak:* sulphur to chrome yellow, smooth, soft. *Broth:* clear, with abundant yellow sediment. *Potato:* sulphur to chrome yellow, raised; sometimes limited growth.

Sarcina aurantiaca Flügge. Spheres developing packets in all media. *Gelatin plate:* small, circular, dark yellow entire margin, sinking into the medium. *Gelatin stab:* infundibuliform liquefaction. *Agar streak:* slightly raised, orange-yellow to orange-red, soft, smooth. *Broth:* flocculent turbidity, with abundant sediment. *Potato:* raised, yellow-orange, glistening to dull granular.

Family 4.—Bacteriaceae.

Serratia marcescens (Bizio), formerly *Bacterium prodigiosum*, Lehmann and Neumann. *Coccobacteria:* 0·5 by 0·5 to 1·0 micron, occurring singly and occasionally in chains of 5 or 6 elements. Motile, with four peretrichous flagella. *Gelatin plate:* thin, slightly granular, grey, becoming red; circular, with slightly undulate margin; liquefies the medium rather quickly. *Gelatin stab:* infundibuliform liquefaction; sediment in liquefied medium usually red on top, white in the depth. *Agar plate:* circular, thin, granular, grey, becoming red. *Agar streak:* white, smooth, moist layer, taking on an orange-red to fuchsin colour in three or four days, sometimes with metallic lustre. *Broth:* turbid; may form a red ring at surface or slight pellicle, and grey sediment. *Litmus milk:* a red surface growth develops. *Potato:* at first, a white line appears, which rapidly turns red; the growth is luxuriant and frequently shows a metallic lustre.

Serratia lactica, Bergey et al.; formerly *Bacillus lactis erythrogenes* (Hueppe). Rod: 0·4 to 0·5 by 1·0 to 1·4 microns, occurring singly; non-motile. *Gelatin plate:* small, circular, greyish-white, later yellow, sinking into the medium; medium rose colour. *Gelatin stab:* slow liquefaction at the surface, the liquid becoming red, with yellow sediment; the solid portion assumes a weak rose colour. *Agar streak:* moist, fairly luxuriant, yellow growth, the medium assuming a rose to wine colour. *Broth:* turbid, yellow. *Litmus milk:* acid; slow coagulation, having a clear fluid which becomes

TABLE VI TABULAR KEY TO BACTERIACEAE. (For abbreviations see Table V)

Flagella	Pigment: Gel. or Agar	Pigment: Potato	Stab Liquefaction	Milk: Coagulation	Milk: Peptonization	Milk: Litmus	Sugar: Dextrose	Sugar: Lactose	Sugar: Sucrose	Broth	Oxygen required	Indol	Nitrates	Gram	Opt. Temp.	H_2S	*Bacteriaceae*
P	*R*	*R*	*I*	+	–	*R*	*A.G*				±	–	*Rd*	–	25–30		Serratia marescens
–	*Y + R*	*Y*	*C*	+		*R + B*	–	–	–		±	–	*Rd*	–	37		Serratia lactica
+	*Gr*	*Gr*	+	+	+	*B*	*A*				±	–	–	–	30		Flavobacterium synxanthum
–	*Br*	*R.Br*	*C*	–	–	–	–				±	–	–	–	30–35		Flavobacterium brunneum
T	*Y*	*G + Y*	–	–			*A*	*A*			±	–	–	–	30		Flavobacterium turcosium
T	*Y*	*Br*	–				*A*				±	–	*Rd*	–	30		Flavobacterium cereviseae
P	*V*	*V*	*I*		+	*B*					±	–	–	–	25–30		Chromobacterium violaceum
T	*B.Gr*	*B.Gr*		+			–	–	–		±	+	–	–	30		Chromobacterium coeruleum
T	*G + Y*	*Br*	+	+	+	*B*	–	–	–		±	–	*Rd*	–	37		Pseudomonas aeruginosa
T	*G.Gr*	*Br*	*I*	–		*B*	*A*				+	–	*Rd*	–	20–25		Pseudomonas fluorescens
–	–	*Br*	–	–	–	–					±	–	–	–	25		Psuedomonas non-liquefaciens
P	*Br*	*Br*	–			*B*					±	–	–	–	25		Cellulomonas ferruginea
P	–	–	–	–	–	–					±	–	*Rd*	–	25		Achromobacter centropunctatum
P	–	–	–	–	–	–					±	–	*Rd*	–	25		Achromobacter stutzeri
P	–	–	–	–	–	–					±	–	–	–	25–30		Achromobacter agile
P	–	–	–	–	–	–					±	–	*Rd*	–	30		Achromobacter hartlebii
P	*Gr*	*Y.Br*	*S – I*	–	–	–					±	–	–	–	25–30		Achromobacter gasoformans
P	–	–	*C*	–	–	–					±	–	*Rd*	–	25		Achromobacter dendriticus
–	–	–	–	–	+	*R*					±	–	*Rd*	–	25		Achromobacter nitrovorum
T	–	*Br*	±	–	–	(*B*)					±	–	–	+	20		Spirillum volutans

blood red in colour; the reaction becomes alkaline. *Potato:* yellow, with sometimes a slight pink tinge, the medium becoming dark.

Flavobacterium synxanthum (Ehrenberg), Bergey et al.; formerly *Bacillus synxanthus,* Hohl. Rods: 0·8 by 1·9 to 3·0 microns, occurring singly; motile. *Gelatin plate:* thin, bluish grey, glistening. *Gelatin stab:* liquefied. *Agar plate:* large, spreading, transparent. *Agar streak:* white, becoming thick, brownish. *Broth:* turbid, with grey pellicle and sediment. *Litmus milk:* coagulated; peptonized; canary yellow; becoming alkaline, ropy. *Potato:* dirty grey, glistening.

Flavobacterium brunneum (Copeland), Bergey et al.; formerly *Bacillus brunneus,* Copeland. Rods: 0·5 by 1·0 micron, occurring singly and in pairs; non-motile. *Gelatin plate:* small, flesh-coloured to brown. *Gelatin stab:* brown surface growth; crateriform liquefaction. *Agar streak:* very thin, glistening, greyish-yellow, filiform. *Broth:* clear. *Potato:* reddish-brown streak.

Flavobacterium turcosum (Zimmermann), Bergey et al.; formerly *Bacillus turcosus,* Zimmermann. Rods: 0·2 to 0·3 by 0·3 to 1·5 microns, occurring singly; motile, possessing a single polar flagellum. *Gelatin plate:* small, translucent, intense yellow. *Gelatin stab:* small, yellow, convex surface growth, with slight greenish tint; no liquefaction. *Agar streak:* scanty, intense yellow streak. *Broth:* slightly turbid, with yellow sediment. *Potato:* scanty, dry, glistening, greenish yellow.

Flavobacterium cereviseae (Fuhrmann). Rods: straight and slightly curved, 0·6 by 1·5 to 2·0 microns, occurring singly and in chains; motile, possessing four to six polar flagella. *Gelatin plate:* circular, white, slightly contoured, becoming brownish-yellow. *Gelatin stab:* slight yellowish growth in stab; no liquefaction. *Agar plate:* thin, spreading, contoured. *Agar streak:* moist, glistening, thin, whitish-yellow, spreading, contoured. *Broth:* turbid, with greyish-white pellicle and large amount of sediment. *Potato:* brown spreading growth.

Chromobacterium violaceum (Bergonzoni), Bergey et al.; formerly *Bacillus violaceus,* Bergonzoni. Slender rods: 0·8 to 1·0 by 2·0 to 5·0 microns, occurring singly and in chains; motile, possessing peritrichous flagella. *Gelatin plate:* circular, grey, entire margin, assuming a violet colour in the centre. *Gelatin stab:* infundibuliform liquefaction with violet sediment in fluid. *Agar plate:* whitish, flat, glistening, moist, becoming violet. *Agar streak:* deep, violet, spreading growth. *Broth:* slightly turbid, with violet ring and ropy sediment. *Litmus milk:* violet pellicle; digestion; alkaline. *Potato:* limited, dark violet growth.

Chromobacterium coeruleum (Voges), Bergey et al.; formerly *Pseudomonas coerulea*, Chester. Rods: 0·8 by 1·0 to 1·4 microns, occurring singly; motile by polar flagella. *Gelatin plate:* bluish grey. *Gelatin stab:* slight surface growth; slow infundibuliform liquefaction. *Agar streak:* bluish grey, moist, glistening. *Broth:* greyish pellicle. *Litmus milk:* coagulated with sky-blue cream layer. *Potato:* greyish blue to blue-green growth, darkening with age.

Pseudomonas aeruginosa (Schröter), Migula; formerly *Bacterium pyocyaneum*, Lehmann and Neumann. Rods: 0·5 to 0·6 by 1·5 microns, occurring singly, in hairs, and short chains; motile, possessing one to three polar flagella. *Gelatin plate:* yellowish or greenish-yellow, fringed, irregular, skein-like, granular, rapidly liquefying. *Gelatin stab:* rapid liquefaction; the fluid assuming a yellowish-green or bluish-green colour. *Agar plate:* large, spreading, greyish with dark centre and translucent edge, irregular; the medium assumes greenish colour. *Agar streak:* abundant, thin, white, glistening, the medium turning green to dark brown or black, fluorescent. *Broth:* marked turbidity, with thick pellicle and heavy sediment, the medium becoming yellowish green to blue, with fluorescence, later brownish. *Litmus milk:* a soft coagulum is formed, with rapid peptonization and reduction of litmus; reaction alkaline. *Potato:* Luxuriant, dirty brown, the medium becoming dark green.

Pseudomonas fluorescens (Flügge), Migula; formerly *Bacillus fluorescens liquefaciens*, Flügge. Rods: 0·3 to 0·5 by 1·0 to 1·8 microns, occurring singly and in pairs; motile, possessing a polar flagellum. *Gelatin plate:* circular, with greenish centre; lobular, liquefying quickly. *Gelatin stab:* infundibuliform liquefaction, with whitish to reddish-grey sediment. *Agar streak:* abundant, reddish layer, becoming reddish grey; the medium shows greenish to olive-brown coloration. *Broth:* turbid, flocculent, with yellowish-green pellicle and greyish sediment. *Potato:* thick, greyish-yellow, spreading, becoming light sepia brown in colour.

Pseudomonas non-liquefaciens (Bergey et al.); formerly *Bacillus fluorescens non-liquefaciens*, Eisenberg. Short, slender rods, with rounded ends, occurring singly; non-motile. *Gelatin plate:* fern-like surface colonies; medium around colonies has a pearly lustre. *Gelatin stab:* surface growth has fluorescent shimmer; no liquefaction. *Agar streak:* greenish layer. *Broth:* turbid, fluorescent. *Potato:* diffuse, brownish layer; medium acquires a greyish-blue colour.

Cellulomonas ferruginea (Rullmann), Bergey et al.; formerly *Bacillus ferrugineus*, Rullman. Rods: 0·5 to 0·8 by 1·5 to 2·0 microns, occurring singly; motile, possessing peretrichous flagella.

Gelatin plate: brown, the pigment diffusing into the medium. *Gelatin stab:* no liquefaction. *Agar streak:* rusty brown streak. *Broth:* turbid. *Potato:* rusty-brown streak.

Achromobacter centropunctatum (Bergey et al.); formerly *Bacillus centropunctatus*, H. Jensen. Rods: 0·3 by 0·5 micron, occurring singly; encapsulated; motile, possessing peritrichous flagella. *Gelatin plate:* thin, greyish, moist, soft, flat, glistening. *Gelatin stab:* white, fimbriate, spreading surface growth; no liquefaction. *Agar plate:* grey, moist, glistening, slimy. *Agar streak:* thin, greyish, moist, centre raised, becoming thick. *Broth:* turbid, with pellicle. *Potato:* greyish, slimy.

Achromobacter stutzeri (Lehmann and Neumann). Rods: 0·5 by 2·0 to 4·0 microns, occurring singly and in short chains; motile, possessing peritrichous flagella. *Gelatin plate:* small, white, entire. *Gelatin stab:* no liquefaction. *Agar plate:* small, bluish white, homogeneous, entire. *Agar streak:* greyish-white, slimy, undulate. *Broth:* turbid. *Potato:* white, slimy.

Achromobacter agile. Rods: 0·3 to 0·5 by 1·0 to 2·5 microns, occurring singly; motile, possessing peritrichous flagella. *Gelatin plate:* small, white, homogeneous, entire. *Gelatin stab:* greyish-white surface growth; no liquefaction. *Agar plate:* small, white, slimy. *Agar streak:* limited, greyish, white, slimy. *Broth:* turbid, with pellicle. *Potato:* limited, greyish-white.

Achromobacter hartlebii, Bergey et al.; formerly *Bacillus hartlebii*, H. Jensen. Rods: 0·7 by 2·0 to 3·0 microns, occurring singly; motile, possessing peritrichous flagella. *Gelatin plate:* small, white, translucent, entire, to erose margin. *Gelatin stab:* white, slimy growth on surface; no liquefaction. *Agar plate:* small, white, translucent, slimy, entire. *Agar streak:* thick, greyish-white, moist, glistening, watery. *Broth:* turbid, with pellicle. *Potato:* greyish-white, moist.

Achromobacter gasoformans (Eisenberg) Bergey et al.; formerly *Bacillus gasoformans*, Eisenberg. Small rods, occurring singly; motile, possessing peritrichous flagella. *Gelatin plate:* circular, grey. *Gelatin stab:* saccate to infundibuliform liquefaction, with much gas formation. *Agar plate:* circular, white, marmorated. *Agar streak:* Dirty-white, smooth, glistening. *Broth:* turbid. *Potato:* slimy, yellowish, becoming brownish.

Achromobacter dendriticus (Bordoni-Uffreduzzi), Bergey et al.; formerly *Bacillus dendriticus*, Bordoni-Uffreduzzi. Rods: 0·5 to 0·8 by 0·8 to 2·0 microns, occurring singly; motile, possessing peritrichous flagella. *Gelatin plate:* large, white, raised, moist, glistening, amoeboid. *Gelatin stab:* slow, crateriform, liquefaction.

Agar streak: thin, irridescent layer. *Broth:* turbid, with tough, adherent pellicle. *Potato:* white, moist, glistening, rough.

Achromobacter nitrovorum (H. Jensen), Bergey et al.; formerly *Bacterium nitrovorum*, H. Jensen. Rods: 0·5 by 0·5 by 1·0 micron, occurring singly; non-motile. *Gelatin plate:* white, slimy, entire. *Gelatin stab:* white, moist, glistening surface growth; no liquefaction. *Agar plate:* greyish-white, slimy. *Agar streak:* greyish, moist, slimy, entire. *Broth:* turbid. *Potato:* dirty-white, slimy.

Spirillum volutans (Ehrenberg). Spirals: 2 to 3 by 30 to 50 microns, with slightly attenuated ends; motile, possessing a bundle of three to eight flagella at each pole; dark granules of volutin in the cytoplasm. *Gelatin plate:* grey, smooth, glistening, entire. *Gelatin stab:* porcelain-white, crumpled surface growth; slight growth in stab; slow liquefaction. *Broth:* turbid. *Potato:* dry, brown streak.

FAMILY 5.—BACILLACEAE (Table VII).

Bacillus cohaerens (Gottheil). Rods: 0·35 to 0·55 by 0·75 to 2·25 microns, occurring singly and in pairs; motile, with peritrichous flagella. Spores central, 0·8 to 1·0 by 1·7 to 2·2 microns. Cells store glycogen as reserve material. *Gelatin plate:* white, irregular, lobed. *Gelatin stab:* irregular, whitish surface growth; slow crateriform liquefaction. *Agar plate:* circular, yellowish-white, folded. *Agar streak:* thin, smooth, glistening, homogeneous, slimy, becoming yellow. *Broth:* turbid, with dense, flocculent pellicle. *Potato:* thin, moist, yellow, spreading.

Bacillus simplex (Gottheil). Rods: 0·9 by 3·0 to 5·0 microns, occurring singly, in pairs, and in chains; motile, with peritrichous flagella. Spores central, 0·8 by 1·4 to 1·7 microns. Cells store glycogen as reserve material. *Gelatin plate:* circular, whitish, entire. *Gelatin stab:* whitish surface growth; liquefaction stratiform. *Agar plate:* thin, translucent, amoeboid. *Agar streak:* thin, spreading, translucent, wrinkled, adherent, becoming yellowish. *Broth:* slightly turbid. *Potato:* thick, moist, slimy, yellowish-brown.

Bacillus lutens (Smith and Baker). Rods: 1·4 to 1·6 by 6·0 to 8·0 microns, occurring singly and in chains. Spores central, 0·8 to 1·4 by 1·0 to 2·6 microns; motile, with two or four peritrichous flagella. *Gelatin plate:* circular, yellow, with three concentric zones, the central one showing most pigment. *Gelatin stab:* slow crateriform liquefaction. *Agar plate:* circular, yellow, homogeneous, entire. *Agar streak:* spreading, bright yellow, becoming brownish-red, glistening. *Broth:* turbid. *Potato:* deep yellow, moist growth.

Bacillus mycoides (Flügge). Rods: 0·8 by 2·0 to 4·0 microns, occurring in long chains. Motile, with peritrichous flagella. Spores

TABLE VII. TABULAR KEY TO BACILLACEAE (For abbreviations see Table V)

Spores : Position	Spores : Swelling of Rods	Flagella	Pigment : Gel. or Agar	Pigment : Potato	Stab Liquefaction	Milk : Coagulation	Milk : Peptonization	Milk : Litmus	Sugar : Dextrose	Sugar : Lactose	Milk : Sucrose	Broth	Oxygen requirement	Indol	Nitrates	Grammes	Opt. Temp.	H_2S	*Bacillaceae*
C	+	*P*	–	*Y*	*C*		+	*R*	*A*		*A*		+	–	–	+	30		Bacillus cohaerens
C	+	*P*	–	*Y.Br*	*H*	–	+		*A*				±	–	*Rd*	+	30		Bacillus simplex
C	+	*P*	*Y*	*Y*									±	–	–	+	35		Bacillus lutens
C	+	*P*	–	–	*S*				*A*		*A*		±	–	–	+	30		Bacillus mycoides
C	+	*P*	–						*A*	*A*			±	–	*Rd*	+	37	±	Bacillus subtilis
C	+	*P*	–	*Y.Gr*					*A*	*A*			±	–	*Rd*	+	30–35		Bacillus ellenbachensis
C	–	*P*	–		*H*	+	–						±	–	–	+	30		Bacillus teres
C	–	*P*	–	–	*C*	+	+		*A*		*A*		±	–	–	+	30		Bacillus vulgatus
C	–	*P*	–	–	*C.S*	–	+		*A*	*A*	*A*		+	–	–	+	35		Bacillus megatherium
C	–	*P*	–	*Br*	*C.S*	–	+		*A*		*A*		±	–	–	+	30	±	Bacillus mesentericus
C	–	*P*	*Br.Y*	*Gr*	–								±	–	–	+	35		Bacillus sphaericus
T	+	*P*	*C*	–	*I*	+	+	*R*	*A.G*	*A.G.*	*A.G*		±	–	*Rd*	+	35		Bacillus asterosporus
T	+	*T*	–	–	–	+			*A*	*A*			±	–	–	+	37		Bacillus macerans
C	–	–	(*Gr*)	*Gr*	*I.H*	–	+	*B*	*A*		*A*		±	–	*Rd*	+	30		Bacillus panis
C		–	(*G*)	*Y*									±	–	–	+	62		Bacillus thermophilus
C	–	*P*	–	–	–	+		*R*					–	–	*Rd*	+	30–40		Clostridium butyricum
C	–	–	–	–	+	+		*R*	*A.G*	*A.G*	*A.G*		–	–	–	+	37		Clostridium welchii
T	+	*P*	–	–	±	±	+		–	–	–		–			+	37		Clostridium tetani

central, 0·8 by 1·4 to 2·2 microns. Cells store fat as reserve material. *Gelatin plate:* whitish, filamentous, fimbriate. *Gelatin stab:* arborescent growth in stab; saccate liquefaction. *Agar plate:* greyish, spreading, rhizoid. *Agar streak:* whitish, soft, glistening, rhizoid, becoming dull. *Broth:* slightly turbid, with wrinkled pellicle. *Potato:* whitish, homogeneous to granular, becoming brownish.

Bacillus subtilis (Ehrenberg), Cohn. Rods: 0·8 by 1·5 to 4·0 microns, occurring singly and in chains; motile, with peritrichous flagella. Spores central, 0·5 to 0·75 micron in size. Cells store glycogen as reserve material. *Gelatin plate:* circular, whitish, entire, becoming creamy white, spreading, filamentous, liquefying. *Gelatin stab:* whitish surface growth; liquefaction stratiform to saccate. *Agar plate:* spreading, greyish, amoeboid, with crenate margin. *Agar streak:* thin, greyish-white, membraneous, glistening, spreading, adherent. *Broth:* turbid, with fragile pellicle and greyish sediment. *Potato:* luxuriant, warty, grey, becoming pink with vesicles over surface.

Bacillus ellenbachensis (Stutzer). Rods: 0·5 to 2·0 by 2·0 to 5·2 microns, occurring singly, in pairs, and chains. Motile, with peritrichous flagella. Spores central, 0·8 by 1·7 to 2·2 microns. Cells store fat and volutin as reserve materials. *Gelatin plate:* circular to irregular, whitish, margin fimbriate. *Gelatin stab:* liquefaction napiform, becoming saccate. *Agar plate:* greyish-white, greasy, glistening, circular, with indefinite margin. *Agar streak:* smooth, whitish, glistening. *Broth:* turbid, with greyish sediment. *Potato:* Yellowish-grey, flat with undulate margin.

Bacillus teres (Neide). Rods: 0·9 to 1·2 by 2·0 microns, occurring singly, in pairs, and short chains. Motile, with peritrichous flagella. Spores central, 0·8 to 1·2 by 1·2 to 2·0 microns. Cells store glycogen as reserve material. *Gelatin stab:* greyish-white surface growth; liquefaction stratiform. *Agar plate:* bluish-grey, finely granular. *Agar streak:* Greyish to white, glistening, slightly wrinkled. *Broth:* turbid, with heavy sediment.

Bacillus vulgatus (Trevisan). Rods: 0·5 by 2·0 to 3·0 microns, occurring singly and in pairs. Motile, with peritrichous flagella. Spores central, 0·5 by 1·2 microns. *Gelatin plate:* circular, homogeneous, refractive. *Gelatin stab:* liquefaction crateriform. *Agar plate:* circular, greyish, refractive, irregular to entire. *Agar streak:* moist, white or cream-white, slightly spreading, slightly raised; non-adherent. *Broth:* turbid, with thin grey pellicle, becoming clear. *Potato:* thick, white to pink, wrinkled, folded, becoming brownish.

Bacillus megatherium (De Bary). Rods: 2·5 to 3·0 by 3·5 to 4·0 microns, occurring singly, in pairs, and short chains. Motile. with ten to twelve peritrichous flagella. Spores central, 0·75 to 1·25 by 1·5 to 2·0 microns. The cells store fat as reserve material. *Gelatin plate:* greyish-white, raised, glistening, entire. *Gelatin stab:* greyish-white surface growth; small, white colonies in stab; liquefaction crateriform to saccate. *Agar plate:* circular, thick, white to cream colour, entire. *Agar streak:* dirty white, smooth glistening, slimy, the medium becoming brownish; old cultures become yellowish-red, with pellucid spots over the surface. *Broth:* turbid, with flocculent surface growth. *Potato:* thick, white, mealy, becoming pale yellow.

Bacillus sphaericus (Neide). Rods: 0·9 to 1·3 by 3·5 to 3·8 microns, occurring singly and in pairs. Motile, with peritrichous flagella. Spores spherical, central, 1·2 to 1·5 microns in diameter. Cells store volutin as reserve material. *Gelatin plate:* small, barely visible, circular. *Gelatin stab:* slight surface growth; small, yellowish colonies in stab, becoming arborescent; no liquefaction. *Agar plate:* small, clear, transparent, becoming yellowish-brown. *Agar streak:* thin, transparent layer, becoming yellowish-brown. *Broth:* turbid. *Potato:* thin, greyish layer; slow development.

Bacillus asterosporus (Meyer), Migula. Rods: 1·0 to 1·2 by 3·0 to 6·0 microns, occurring singly and in pairs. Motile, with peritrichous flagella. Spores terminal, 1·0 to 1·5 by 1·7 to 2·5 microns, star-shaped on cross-section. Cells store glycogen and volutin as reserve materials. *Gelatin stab:* liquefaction infundibuliform. *Agar plate:* slightly raised, circular to irregular, yellow, concentric. *Agar streak:* thin, transparent to yellowish-white layer. *Broth:* turbid. *Potato:* white, raised, glistening, with gas bubbles.

Bacillus macerans (Schardinger). Rods: 0·8 to 1·0 by 4·0 to 6·0 microns, occurring singly and in pairs. Motile, with polar flagella. Spores terminal, 1·5 by 2·0 microns. *Gelatin plate:* mall, white. *Gelatin stab:* slight surface growth; no liquefaction. *Agar plate:* grey, circular, contoured. *Agar streak:* barely visible, colourless growth. *Broth:* turbid, with slimy sediment. *Potato:* rapid, heavy growth, with gas formation.

Bacillus panis (Vogel), Migula. Rods: 0·375 to 0·5 by 1·5 to 3·0 microns, occurring singly and in chains. Non-motile. Spores central, 0·375 to 0·5 by 1·0 to 1·25 microns. *Gelatin plate:* greyish, with brown centre. *Gelatin stab:* liquefaction infundibuliform, becoming stratiform. *Agar plate:* small, greyish, slightly irregular, translucent, raised, viscid. *Agar streak:* scanty, slightly raised, greyish, finely wrinkled, translucent, viscid. *Broth:* slightly turbid, with granular pellicle. *Potato:* greyish, finely wrinkled, viscid.

Bacillus thermophilus (Rabinowitsch). Rods, slightly bent, occurring in pairs; non-motile. Spores central. *Agar plate:* greenish, granular, spreading, irregular. *Broth:* becomes alkaline. *Potato:* greyish-yellow, with undulate margin.

Clostridium butyricum (Prazmowski). Rods; 0·75 to 1·0 by 3·0 to 10·0 microns, occurring singly, in pairs, and long chains; may produce slender forms, 0·5 micron in width. Actively motile in young cultures, possessing peritichous flagella. Spores oval, central, 1·0 by 2·0 to 2·5 microns. Cells store glycogen. *Gelatin stab:* no liquefaction. *Plain and dextrose agar streak:* (anaerobic)—greyish, flat, moist, spreading, with irregular, lacerate margin; no surface growth aerobically. *Agar shake cultures:* compact with dense central portion and floccose, hazy periphery, 2 to 3 mm. in diameter. *Anaerobic agar stab:* granular, filiform growth. *Dextrose broth:* turbid. *Potato:* (with chalk)—shows thin, spreading, barely visible layer, forming later yellowish, raised points. The medium becomes soft and friable, with gas bubbles.

Clostridium welchii (Migula), Holland. Rods: short, thick; 1·0 to 1·5 by 4·0 to 8·0 microns, occurring singly and in pairs, less frequently in short chains; non-motile. Spores oval, central; encapsulated. *Gelatin stab:* liquefied and blackened. *Agar plate:* circular, moist, slightly raised, opaque centre, entire. *Broth:* turbid, peptolytic. *Potato:* thin, greyish-white streak. Gas in subtended liquid.

Clostridium tetani (Nicolaier), Holland. Rods: 0·4 to 0·6 by 4·0 to 8·0 microns, occurring singly; motile, with peritrichous flagella. Spores spherical, terminal. *Gelatin stab:* slowly liquefied. *Agar streak:* thin, transparent. *Broth:* slightly turbid. Gas.

The bacteria mentioned below are typical species belonging to other orders which are quite frequently encountered in their appropriate environments.

Crenothrix polyspora (Cohn). Long, stiff filaments, unbranched, segmented; 1·5 to 5·2 microns thick; sheath, growing thicker with age, containing iron oxide; vegetating cells × 0·5 to × 4, the thickness of the filament. Reproduction by segmentation of vegetating cells into small round micro conidia; and by breaking up of vegetative cells near tip into large oval macro-conidia. Conidia escape or germinate in the filament. Does not grow on culture media. *Occurrence:* stagnant water, etc.

Rhodocapsa suspensa (Molisch). Rods or filaments 1·8 to 3·5 × 3·5 to 180 microns; capsule; contain red motile granules, and often sulphur granules. Non-motile, not swarming. Form bacteriopurpurin and bacteriochlorin. *Occurrence:* sea-water.

Rhodobacillus palustris (Molisch). Rods: 0·5 × 1·5 to 2·5 microns, rounded ends, and in chains of 2 to 4; motile. Colourless cells, deep red masses; bacterio purpurin and bacterio chlorin produced; grow slowly on agar. *Occurrence:* swamps and streams.

Didymohelix ferruginea (Ehrenberg), Griffith; formerly *Chlamydothrix ferruginea,* Migula. Thin yellow-brown filaments, no obvious cells, colourless young, later brown due to iron oxide; sheath often demonstrated by iodine. Threads 1 micron thick, irregularly bent, may form masses; often form close-set spirals 2 microns thick. *Occurrence:* ferrugineous water.

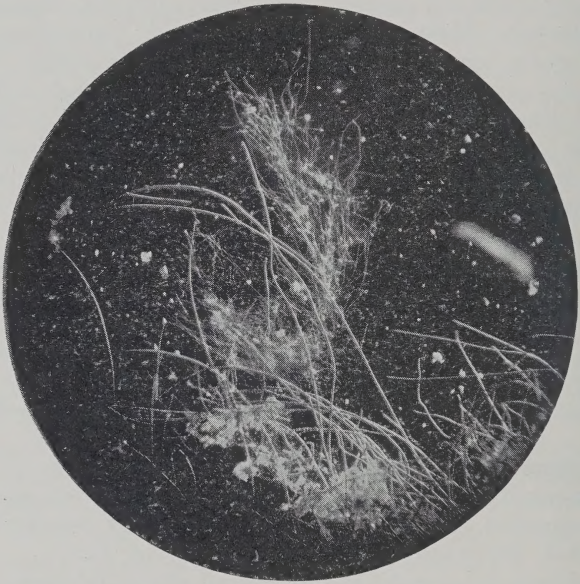


FIG. 184. CLADOTHIX DICHOTOMA AND BEGGIATOA ALBA UNDER DARK GROUND ILLUMINATION (*Jackson*)

Leptothrix ochracea, Kützing (= *Chlamydothrix ochracea,* Migula). Filaments of rods, 0·8 micron thick, delicate sheath; later, thicker and yellow brown, containing iron oxide. Reproduction by formation of short non-motile ovoid conidio spores at ends of filaments. May show pseudodichotomous branching (false branching). Does not grow on usual media. *Occurrence:* ferrugineous water.

Sphaerotilis dichotomus (Cohn), Migula (= *Cladothrix dichotoma,* Cohn). Filaments 2 microns thick, length variable; dichotomous branching, segmenting in sheath to straight or spiral motile bodies, emerging from end or breaking the sheath. *Occurrence:* swamps.

Clonothrix fusca (Schorler). Branching filaments 5 to 7 microns thick, tapering to 2 microns, colourless; later, yellow-brown. Sheath containing iron oxide. Reproduction by longitudinal division of disc cells at tip into small non-motile, round conidio spores. *Occurrence:* water.

Beggiatoa alba (Vaucter), Trevisan. Long threads, sheathless, of flat disc-like cells, not attached, 3 to 4 microns thick. Multiply by breaking up into short segments, which each grow longer. Many highly refractive sulphur granules in cells. Sulphates reduced to S. *Occurrence:* sulphur springs, swamps, forming slimy flakes attached to aquatic plants.

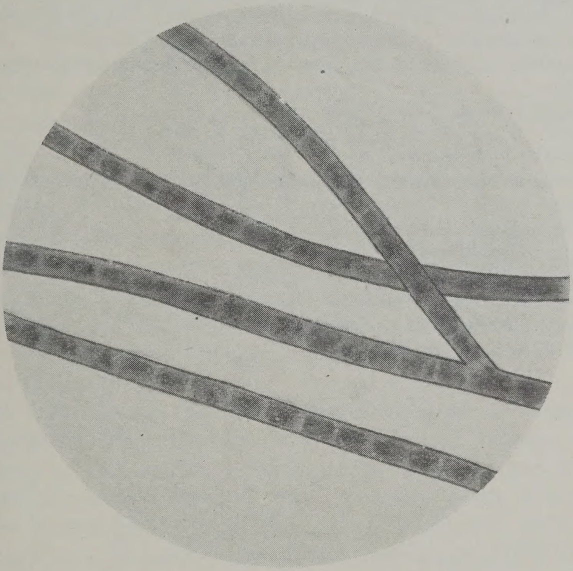


FIG. 185. CLADOTHRIX DICHOTOMA, SHOWING CELLS IN SHEATH, AND PARTICLES OF IRON OXIDE (*Jackson*)

Beggiatoa marina (Molisch). About 300 microns long, 2 to 4 microns thick, otherwise as above. *Occurrence:* on rotting marine algae.

Beggiatoa leptomitiformis (Meneghini), Trevisan. Filaments, 1·8 to 2·5 microns thick. *Occurrence:* as heavy white sediment in sulphur springs, swamps, and sewers.

Thiospora bipunctata (Molisch), Vislonch. Small, slightly bent spirilla, pointed ends, 1·7 to 2·4 × 6·6 to 14 microns. Ends contain volutin granules, centre shows small sulphur granules. Flagellum at one or both ends. *Occurrence:* salt water.

Thiotrix nivea (Rabenhorst), Winogradsky. Filaments 2 to 2·5 microns, tapering to 1·4 to 1·5 × 100 microns (contain sulphur); segmented at tip, forming motile conidia 8 microns long. Slow creeping movement. *Occurrence:* sulphur and stagnant water.

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CHAPTER XV

FUNGI

THE term "fungi" includes a large number of plants of very diverse character, which have this in common: that they do not possess chlorophyll and, in consequence, are unable to build up carbohydrates by means of photosynthesis. They are, therefore, parasites, obtaining their food from other plants or animals; or saprophytes, living upon decaying vegetable or animal matter.

If the large group of yeasts be excluded, there remains only a small number of fungi which are directly useful to man. The major proportion of these organisms, on the contrary, causes an annual loss represented by a very considerable sum of money, because of the damage which they do to wheat, trees, textile materials, rubber, food, and other industrial products. There is probably no organic matter, vegetable or animal, which is free from attack by some species of the fungi.

Certain members of the Mucor family contain enzymes, which enable them to convert starch into sugar, thus enabling starchy materials to be used as raw material for the manufacture of alcohol. A member of the Aspergillus family, *A. oryzae,* finds an important use in the manufacture of diastase, or malt extract; it is allowed to grow on bran or a similar substratum, and the growth is extracted with water. Other Aspergilli produce acid from sugar; thus, *A. niger* forms abundant oxalic acid, and citric acid, from sucrose; whilst Wehmer obtained a patent for the commercial production of fumaric acid by a variety of this species. *A. niger* also forms gallic acid from gall nuts, a process which is in use commercially; this property is, however, more prominent in the leather industry, where its tolerance for tannin materials makes it a serious cause of loss by mildewing leather. Several Aspergilli also are able to digest cellulose, a valuable power in a soil organism, but a serious property in a mould which often infects cotton goods. Flavour is imparted to coffee on a commercial scale by *A. ochraceus,* whilst certain Penicillia are employed in the ripening of special cheeses, e.g. *P. Gorgonzolae.*

As a class, however, fungi suffer from the commercial disadvantage that theirs is a surface action, requiring the material treated to be spread out, for example, on trays, in order to expose large areas. Few fungi are able to work in liquid media in the way in which industrial fermentations are carried out, using bacteria or yeasts. In consequence, liability to contamination by other fungi than the selected organism is very great, and it is difficult to control the conditions of growth in order to confine the action to the desired species.

Against the few useful activities of the fungi is to be set the great losses caused by the growth of moulds and mildews on an astonishing variety of substrata; indeed, to use an expression of Thom and Church, they are "omnivorous." The members of the Aspergilli and Penicillia alone, which so commonly infect starchy materials, such as food, or occur on textile materials, causing damage by the formation of coloured patches or acidity, cause a great annual loss. Tobacco is liable to moulds, *A. niger* and *A. fumigatus* being often noted. The Boll disease of cotton plants, a serious menace to the cotton industry of America, for which the Boll weevil is primarily responsible, is actually caused by a fungus similar to the yeasts, which is carried by the weevil. Dry rot is caused by *Merulius lacrymans*, a highly specialized and very widely spread fungus. In agriculture, mould and mildew diseases are extraordinarily common, and very difficult to deal with; examples are the so-called "rust" on wheat, due to *Puccinia graminis* and *Puccinia bromina*; and potato blight, caused by *Phytophthora infestans*.

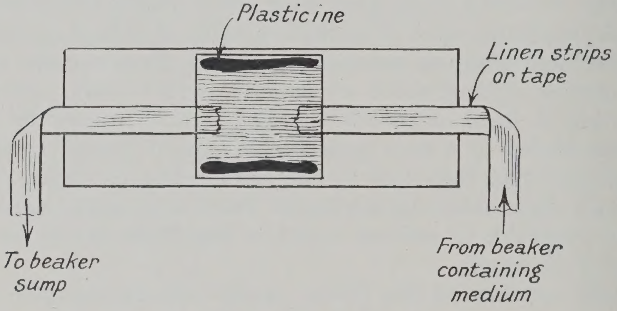

FIG. 186. RUNNING WATER CULTURE

The chief reason for the widespread nature of fungal infection is that they produce an incredible number of spores; it is no exaggeration to say that all organic material is normally heavily infected with fungal spores, which are a potential source of damage, awaiting only the proper conditions for growth—namely, the requisite hygroscopicity of the material, the correct temperature, and a satisfactory *pH* value of the substratum—before developing with extraordinary rapidity. A single giant puff ball may produce seven million million spores; and, though this is exceptional, there is no fungus which does not display an astoundingly prolific spore production. The spores are extremely resistant to wide ranges of temperature and dryness, and are often motile in liquid media, or are so light that they are easily disseminated by the lightest air current. An average mildew spore is only perhaps ten times the size of an average bacillus.

It is evident that it is not industrially practicable to prevent infection, and it is no less impracticable to sterilize and to keep sterile industrial quantities of material. The methods available for the control of fungi are, therefore, limited to the addition of antiseptics, and to the avoidance of such conditions of moisture and temperature as favour growth.

It is necessary, on the other hand, when a fungus is to be employed for a specific purpose, to ascertain the best conditions for growth, and the most practical means of excluding and of retarding the growth of less suitable organisms.

In industrial work, the chief reason for wishing to identify an organism is, of course, in order to open up the information collected by other workers concerning its properties. At the present time, the field has been examined so irregularly in its commercial aspects, that the exact identification of a mildew may not enable any information of value to be obtained. It is, however, a comparatively simple matter to discover to what group an organism belongs, and to determine those characteristic reactions which have a bearing upon industry.

The general methods of working are as described in Chapter XIII with the following modifications: yeasts are grown in liquid media only, as a rule, and the remaining fungi are examined as surface growths on agar plates or streaks. A large number of media have been recommended; of these, prune juice agar is excellent for preliminary work, though wort agar (prepared from solid dried malt extract) or potato agar may be preferred at times. For the final identification these media may be employed, or the Dox-Czapek agar may be substituted. Sugar gelatine is of use in examining the action on protein media. Plain pastes made of starch or flour have their special uses, and bread is often employed also. For examining the behaviour towards cellulose, a tube of sterile Dubos medium, containing a strip of filter paper, is inoculated with the microorganism, and left for some weeks; or Omelianski's medium may be employed, as for bacteria. The milk tube has been used by some workers. Pure cultures are most easily obtained by cutting off a sporing head with a pair of fine-pointed scissors, or fine forceps, transferring this to the centre of a Petri dish containing the medium upon which growth is to be carried out. (See pages 370, 371, 372.)

Mounting may be carried out by transferring a little of a colony on the point of a needle to a drop of water or dilute glycerine on a slide. Lacto phenol cotton blue is an excellent permanent mountant, combining the functions of a fixative preservative mountant and stain; the cotton blue is absorbed from the medium (No. 18).

Pure cultures may be preserved for reference on agar slopes at a temperature of about 5–10° C., without the necessity for frequently making new tubes; or they may be kept at the laboratory temperature if constant observation is maintained.

A useful method of observing the whole structure of a mould is given by Henrici. Two thin rolls of plasticine are spread on a slide, supporting a cover glass a half millimetre from it. The intervening space is half filled from a capillary pipette with an agar medium, which has been inoculated with spores of the fungus. The slide is incubated in a Petri dish in the bottom of which is some wet sterile cotton wool. (Fig. 187.)

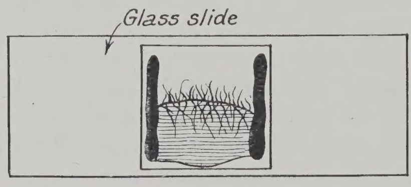

FIG. 187

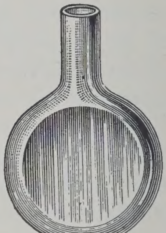

FIG. 188

A convenient method which serves a similar purpose, is to make a streak culture at the bottom of a flat-walled bottle (Fig. 188).

The accepted division of fungi is into four main groups, based largely upon the method of spore formation, the type of sex organs (if present), and the character of the mycelium. These groups are Phycomycetes, Ascomycetes, Basidiomycetes, and the Fungi Imperfecti. The following brief summary follows the classification by Vaughan and Barnes—

PHYCOMYCETES. As a group, these organisms bear some resemblance to green algae, and, indeed, at one time they were supposed to have been derived from the algae, though the modern view is that they have a common parentage. The group, as a whole, possesses large sex organs, thick-walled zygotes appearing shortly after fertilization. The mycelium is usually aseptate. The group is divided into Archimycetes, in which the mycelium is rudimentary or absent; and the Zygomycetes, which have a well-developed mycelium, and which includes the important Mucorales (discussed later in this chapter), sex reproduction being by means of the zygospore.

Ascomycetes. The mycelium, which is normally septate, is not wide spreading like the Phycomycetes, and a definite fruit body is usually formed. There are three subdivisions: Plectomycetes; the ascocarp, if present, shows no definite opening; the group including the Yeasts, the Aspergilli, and the Penicillia, all of which are dealt with in the following pages. The remaining two subdivisions are of little interest from the present standpoint; they are: Discomycetes (ascocarp wide open when ripe) and Pyrenomycetes (ascocarp flask-shaped, opening by an astiole, when ripe).

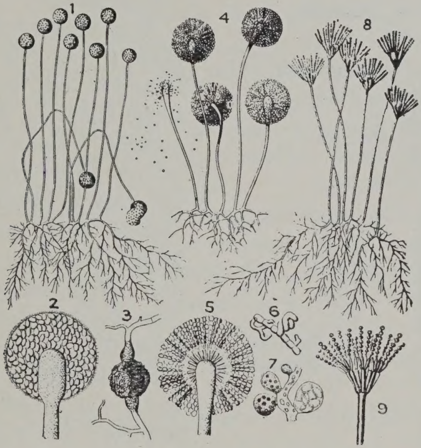

Fig. 189. Fungus Types

Mucor mucedo—asexual spores in sporangium (2) borne on conidiophores (1) with sexual zygospore formation shown at (3).
Aspergillus niger—longitudinal section of head (5); general growth (4).
Penicillium crustaceum—head (9); general growth (8).
Ascospore formation is illustrated at (7).

Basidiomycetes. The members bear a closer relationship with the Ascomycetes than to the Phycomycetes; indeed, this and the preceding group have often been classed together as sub-groups of a larger class termed Eumycetes (e.g. in Strasburger). It contains the important group of plants known as "rusts." The mycelium is usually septate.

Fungi Imperfecti. The name arises from the fact that the members do not show the so-called perfect sexual cycle, which includes the ascus, basidium, or the zygote. Recent work, however, has shown that sexuality in the fungi is a very complex matter; there may actually be four "sexes," which can conjugate in pairs, though the two pairs are intersterile. The mycelium is often not homogeneous, and may be either homothallic, when the whole of it is sexually equivalent, or heterothallic, when certain portions exhibit different sexual tendencies from the rest. In many older works, some members of the Aspergilli and other recognized groups were classed amongst the Fungi Imperfecti, though many modern writers, such as Thom, do not follow this practice.

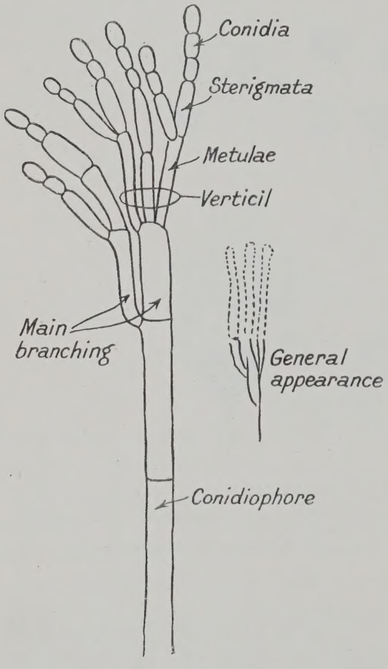

Fig. 190. Penicillium

It is very probable that the majority of the Fungi Imperfecti are only imperfect in the sense that the juncture of the correct strains rarely comes about, but that when this juncture is effected the perfect sexual cycle exhibits itself. Many organisms of considerable importance as mildews and moulds, come under this grouping.

PENICILLIA. *Conidiophores* (stalks) arise out of the mycelial hyphae; in some species a number are fused together to form *coremia*. The (septate) conidiophores branch into from one to three *main branches*, each of which branches further into groups (*verticils*) of smaller branches (*metulae*), which again branch at the tips into *sterigmata* (spore-bearing cells). These abstrict off at the tips, chains of *conidio-spores* (*conidia*). The whole growth from the first branching of the conidiophore upwards is termed the *fructification or penicillus*.

The length of the penicillus given in the following key does not include the conidial chains; the length of the conidiophore is from its inception to the first branching. Descriptions of colonies are all of growth on Dox-Czapek medium, unless otherwise stated.

Key to the Penicillia. The key is compiled from material in *The Penicillia*, by Thom.

1A. Conidia thin walled, no ring or collar at the base; see 2.

1B. Conidia rather thick walled, with thickened ring or collar at the base; never green, aerial hyphae at least partly in trailing and anastomosing ropes or fascicles. Conidiophores very short or absent, conidial apparatus penicillum like, or varying aggregates of branches and sterigmata, or even single sterigmata scattered along the aerial hyphae. Sterigmata taper gradually to the apex or are narrowly tubular; conidia pointed at the apex and truncated at the base. Agents of decomposition in the later stages of decay: *Scopulariopsis.*

P. Brevicaule, Saccardo; now Scopulariopsis brevicaulis (Saccardo), Bainer. *Colonies:* liquefy wort gelatine, *hyphae* creeping, sparingly septate. *Conidiophores:* very short, twice verticillately branched, 3–4 per verticil, end branches (pseudo sterigmata) inverted club-shaped, 14–28μ × 3–4μ. *Fructification:* 20–40μ long. Branches crowded at the base. *Metulae:* 2–3 × 8–17μ. *Sterigmata:* 3–4 × 11–17μ, in groups of 2–5. *Conidia:* lemon-shaped, smooth, later rough (echinate), 4–7 × 5–9μ.

P. Costantini, Bainer; now Scopulariopsis costantini (Bainer), Dale. *Colonies:* cream-orange, velvety; later ropes of hyphae. *Gelatin:* liquefied. *Conidiophores:* 60μ to beginning of fructification; branches 4 × 20μ. *Metulae:* 4 × 10μ. *Sterigmata:* 2–2·5 × 10–12μ; closely-packed verticils, pointed. *Conidia:* colourless, elliptical, thick walled, smooth 2–3·5 × 3·5–6μ. Ring or collar not very noticeable.

2A. Conidial chains not enveloped in slime in laboratory cultures. (See 3.)

2B. Conidial chains soon breaking up; conidia forming a mass enveloped in slime in laboratory cultures, the mucilaginous drops increasing in size with the number of conidia. Later, a fusion of masses on adjacent sterigmata occurs: *Gliocladium.*

P. Roseum, Link; now Gliocladium roseum (Link), Bainer. *Colonies:* floccose, hyphae simple, ropes formed, old cultures show dense masses of sclerotia 1 mm. deep. *Gelatin:* rapidly liquefied. *Conidiophores:* perpendicular branches of aerial hyphae 45–125μ high. *Conidial* fructification: 140μ max., once or twice irregularly, alternately, or verticillately branched. *Sterigmata:* 2–3 × 12–17μ. *Conidia:* colourless, pink in mass, smooth, fine granules content; elliptical, 3–5 × 5–7μ; become aggregated into gelatinous masses.

3A. Sterigmata in verticils (characteristic); conidium-forming points or tubes straight; conidial areas green, blue-green, or grey-green when growing, rarely white, yellow or red: *Penicillium.* (See 4.)

3B. Sterigmata irregular groupings, partly of fertile branchings partly in verticils; conidium-forming tips usually long, slender, curved or bent; conidial areas never green: *Paelomyces.*

P. divaricatum, Thom; now Paecilomyces varioti, Bainer. Bean agar *cultures*: yellow-brown, never green, trailing hyphae, powdery when old, reverse not coloured. *Gelatin:* not liquefied. *Fertile hyphae:* short, septate, creeping. *Conidial fructification:* characteristic; terminal or on short branches of semi-erect hyphae. Separate sterigmata, verticils, and series of verticils of branches and sterigmata, all found irregularly distributed. *Sterigmata:* 3 × 15–20μ. *Conidia:* long chains, elliptical or fusiform, 2·5–5 × 5–7μ. Yellow-brownish; swell on germination to 10μ, protruding two or more tubes.

4A. Penicilli are typically monoverticillate, borne either on branched conidiophores, or on branches so arranged that each monoverticillate penicillus preserves its distinct identity: *Monoverticillata.* (See 5.)

4B. Penicilli show two or more series of elements (including sterigmata and metulae) without branching of one or more of the series, the branching system being lopsided: *Asymmetrica.* See (6).

4C. Penicilli show one symmetrical verticil of metulae, each bearing symmetrical verticils of sterigmata at its apex: *Biverticillata symmetrica.* (See 7.)

5A. Branching of conidiophores, if present, occasional and irregular. No distinct terminal verticils of metulae or branchlets. *Monoverticillata stricta.*

P. frequentans, Westling. *Colonies:* (Czapek + cane-sugar) blue-green to green, surface growth of short, crowded condiophores and trailing hyphae, narrow white margin in young colonies; reverse, orange-yellow to brown; agar in lighter shades. *Gelatin:* liquefied. *Conidiophores:* 2–3 × 100–125μ, swollen to double diameter at the tip. *Penicillus:* single, dense verticil. *Sterigmata:* 2–3 × 7–10μ. *Conidia:* chains in a solid column often 500μ long; globose, 2·5–3μ, granular contents.

P. Spinulosum, Thom. *Colonies:* deep green, broad spreading, sterile margin when young, aerial portion consisting of conidiophores and aerial hyphae; reverse uncoloured. *Gelatin:* slowly liquefied. *Conidiophores:* 3–3·5 × 150–300μ, apex enlarged to 5μ; single verticil of sterigmata, 2–3 × 9–11μ. *Penicillus:* loose column of chains, 15–30 × 300–500μ. *Conidia:* piriform to globose, 3–3·5 × 3·5–4μ. Thin walled, smooth, later spinulose; yellowish-green, later smoke colour.

5B. Fertile hyphae mostly branching, either one-celled, or long and septate; simple or again occasionally branched; terminal penicillus monoverticillate, but showing no verticils of metulae or branchlets: *Monoverticillata Ramigena.*

P. cyaneum, Biourge. *Colonies:* close felt. *Mycelium:* hyphae 1–2μ, dull blue to grey green; reverse colourless, later pinky. *Conidiophores:* 2 × 100–300μ, separately from substratum or branching from aerial hyphae. *Penicilli:* monoverticillate, with narrow conidial columns. *Sterigmata:* variable length to 20μ, few, crowded. *Conidia:* 2–2·5 × 3–4μ.

6A. Colonies velvety, conidiophores like wheat in a field, but may show a net of aerial hyphae at their base: *Velutina.*

P. Roqueforti, Thom. *Colonies:* velvety, smooth surface, broad spreading; broad white flimsy margin; green conidial areas in radiating lines; 100–300μ deep; reverse, blue-green to black. *Conidiophores:* 4–6 × 100μ from aerial or submerged hyphae, often branched, walls granular and pitted. *Penicilli:* simple, monoverticillate, verticils of metulae and sterigmata. *Sterigmata:* 3 × 12μ. *Conidia:* globose, 4–5μ; chains long, forming fairly compact columns.

P. chrysogenum, Thom. *Colonies:* velvety, later pinkish tufts of hyphae, conidial areas greenish-blue, later violet-brown; 300–400μ deep; reverse yellowish; abundant yellow drops, no odour. *Conidiophores:* mostly from submerged hyphae, 4 × 300μ. *Penicillus:* terminal verticil of metulae, with other branches below, carrying verticils of sterigmata. *Metulae:* 3·5–4 × 10–16μ. *Sterigmata:* 3·5 × 8–9μ. *Conidia:* elliptical × 4μ.

P. Digitatum, Saccardo. *Colonies:* greenish, aromatic odour. *Conidiphores:* 5μ, short. *Penicillus:* 60μ, smooth walls, branches in twos and threes, appressed or divergent; 3–4·5 × 11–28μ in groups of 2, 3, or 4. *Conidia:* great variations, globose or elliptical, 3–6 × 6–21μ.

P. Puberulum, Bainer. *Colonies:* velvety, broadly-spreading thin area gradually covers the whole surface, blue-green, later green; aerial hyphae follow the submerged margin, reverse yellow-green or tan, agar uncoloured, mouldy odour. *Conidiophores:* 3·5–4 × 100–200μ; walls smooth or somewhat pitted. *Penicillus:* one dense column, or several ragged divergent columns; terminal verticil of metulae, with lower branches, all enlarged at the apex. *Sterigmata:* 2–2·5 × 7–10μ. *Conidia:* 3·5–4μ elliptical, later globose.

6B. Colonies are floccose-velvety. The penicillus consists of one or more branches closely compacted at the base, bearing metulae and sterigmata, which are divergent at the apex with chains of conidia either parallel or divergent, but not in columns: *Brevi-compacta.*

P. brevi-compactum, Dierckx. (P. stoloniferum, Thom.) *Colonies:*

grey-green to green, velvety, loose, long stalked, aerial felt 1 mm. high, very narrow white margin. *Gelatin:* quickly liquefied. *Conidiophores:* 4–6μ obscurely pitted. *Penicillus:* short appressed primary branch. *Metulae:* crowded and divergent. *Sterigmata:* crowded. *Conidia:* 3–3·5μ; chains parallel, then tangled.

6C. Colonies are floccose or lanose; hyphae never in ropes or fascicles, but form floccose felts; pencillus consists of the compacted or complex brush of the asymmetrica: *Lanata-typica.*

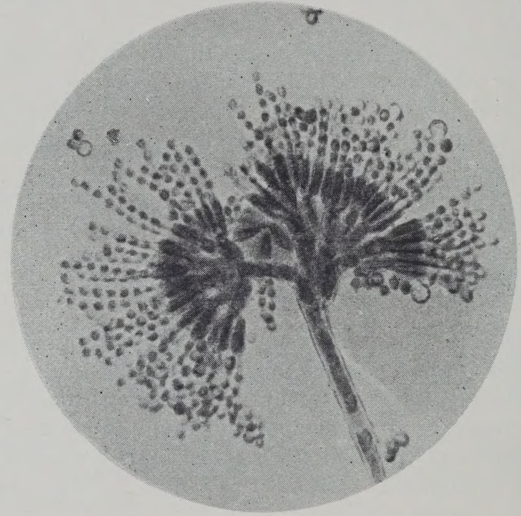

Fig. 191. P. Brevi-compactum × 600 (*Smith*)

P. camemberti,

P. lanosum,

6D. Colonies are lanose; aerial hyphae trailing, not erect; penicillus, a one-sided verticil of divergent and mostly unequal metulae-bearing verticils of sterigmata, giving the effect of a cluster of monoverticillate penecilli: *Lanata-divaricata.*

6E. Colonies are floccose, aerial hyphae partly trailing, branching, and usually anastomising into ropes or funicules, never erect; conidiophores partly rising directly from submerged mycelium, partly from the ropes; penicillus either divaricata (as in Lanata divaricata) or compact (as in Lanata typica): *Funiculosa.*

P. Solitum, Westling. *Colonies:* (Czapek + cane-sugar) blue-green to green, floccose, broad spreading, margin white, ropy, reverse yellow, fragrant odour. *Conidiophores:* smooth. *Conidial fructification:* once or twice verticillately branched, branches appressed.

Sterigmata: 2–3·5 × 8·5–10μ. *Conidia:* smooth, globose-elliptical, 3·3–4μ, granular contents, chains do not easily break up.

6F. Part or all conidiophores aggregated into fairly definite and erect bundles of fascicles or coremia; penicilli asymmetrical; walls of conidiophores pitted or rough. Simple conidiophores may predominate mixed with fascicles, or coremia may predominate: *Fasciculata.*

P. schneggi, Boas. *Colonies:* coremia 2–12 mm. long, later feathery. *Conidial masses:* green; reverse yellow, later reddish. *Sterile hyphae:* 3–4μ diameter. *Conidiophores:* mostly branching from coremia, 4–5μ, forming two equal branches 12–45μ long, then two verticils of metulae, primary 13–21μ long, secondary 7–13μ long. *Sterigmata:* 8–12μ long. *Conidia:* elliptical, 2·5–2·8μ long.

P. expansum (Link), Thom. *Colonies:* (potato agar) grey-green, later brown; concentric zones tufted with coremium-like groups of conidiophores, less than 1 mm. high; broad white margin; reverse brownish. *Gelatin:* liquefied. *Conidiophores:* (*a*) very short lateral branches of aerial hyphae (b.), very long (1 mm.), usually in groups. *Penicilli:* 1–3 main branches bearing verticils of branchlets. *Sterigmata:* 2–3 × 8–10μ in crowded whorls. *Conidia:* form long chains, elliptical 3·3μ.

P. granulatum, Bainer. *Colonies:* (potato agar) yellow-green to grey-brown crowded small coremia, 1–3 mm. high, mixed with floccose hyphae and separate conidiophores.

P. cyclopium, Westling. *Colonies:* zonate when old; velvety, thin margin, centre 500μ deep. *Conidiophores:* crowded, in tufts, no definite coremia, margin white, later blue-grey. Reverse yellowish, later purplish, peculiar odour. *Conidiophores:* rough walled, 3μ. *Penicillus:* 60μ long, main stalk and partly divergent branch, each bearing verticils of metulae, branches in pairs, 2·5–3 × 25–40. *Metulae:* 2–3 × 9–13μ, in 3–4 per group; apex swollen. *Sterigmata:* 2–3 × 7–8μ, verticila of 2–7. *Conidia:* 3–4μ; globose.

P. corymbiferum, Westling. *Colonies:* (prune gelatin) white margin, centre blue-green; surface growth mostly conidiophores in small crowded coremia up to 5 mm. high, feathery branching above. *Gelatin:* slowly liquefied; reverse yellow-orange. *Hyphae:* 2·4–7μ calcium oxalate crystals. *Conidiophores:* 4·2–6 × 45–700μ; walls rough or almost smooth. *Penicillus:* 40–120μ; main stalk, and appressed clavate branch with verticils of metulae and sterigmata at a uniform level, symmetrical. *Metulae:* 3·2–4·5 × 12–16μ; walls rough or smooth. *Sterigmata:* 3 × 8–9·6μ. *Conidia:* 2·6–3·2μ; globose, smooth.

P. claviforme, Bainer. *Colonies:* white or grey. Conspicuous coremia with compact fibrous stalks, up to 2 cm. Also loose floccose hyphae bearing scattered inconspicuous simple penicillate conidial masses. Reverse brown in age. Strong odour. Colourless drops during growth. *Penicilli:* simple, sparingly branched, bearing verticils of a few sterigmata. *Heads:* olive-green, complex hymenium-like masses covered with sterigmata. *Sterigmata:* 2 × 9–10μ, crowded, radiating. *Conidia:* elliptical, 3–3·5 × 4–4·6μ, masses into columns; long chains.

7A. Perithecia and ascospores formed: *Ascogena.* § *P. avellaneum,* Thom and Turesson. *Colonies*: broad spreading, slightly floccose. *Perethecia:* slowly formed together with red aerial hyphae; reverse and agar red. *Conidiophores:* 3–5 × 400μ. *Conidial fructifications:* parallel or tangled chains, 200μ. Fertile branchings: (*a*) terminal crowded, verticil of metulae 3 × 8–10μ, bearing verticils of *sterigmata* 2 × 8–9μ; (*b*) branches from the terminal 10–15μ of conidiophore. *Conidia:* ellipsoid-globose, 2–2·5 × 3–3·5μ, smooth, swelling to 10μ on germination. *Perethecia:* ellipsoidal 300–600μ. *Ascospores:* ellipsoidal, 4–5 × 6·5–8·5μ, walls thick, pitted.

7B. Abundant erect coremia: *Coremigena.* § *P. Duclauxii,* Delacroix. *Colonies:* (potato agar) dark green to olive when old. *Coremia:* short, but on media containing milk or cane sugar, very vigorous growth. *Conidiophores:* short, crowded, arising singly from medium, 10–15μ. *Conidial fructification:* simple, or combined with a lateral branch bearing a whorl of sterigmata. *Penicillus:* up to 100μ long. *Conidia:* elliptical, 2–2·5 × 3·6–4μ, clear green, smooth, later rugulose.

7C. Green conidial areas on yellow or red mycelium: *Luteo-virida. P. pinophilus,* Hedgcock. *Colonies:* (potato agar) green, aerial hyphae studded with yellow granules on acidified media. Reverse and agar deep red. *Conidiophores:* 100–200μ. *Penicillus:* 120μ, single verticil of metulae (2–2·5 × 10–16μ) bearing whorls of *Sterigmata:* (2–2·5 × 13–15μ). *Conidia:* elliptical, 3–3·6 × 2μ, smooth, pale green, chains parallel, but not forming into columns. Produces discoloration of timber.

P. rugulosum, Thom. *Colonies:* yellow-green, later dark green, reverse yellow-orange in spots, agar colourless. *Conidiophores:* 2·5–3 × 100–300μ. *Conidial fructifications:* 100–150μ long, verticillate branches (2·5 × 10–15μ) bearing verticils of sterigmata or metulae, or both. *Sterigmata:* 2 × 9–12μ. *Conidia:* 2·5 × 3·5μ elliptical, green, forming long divergent chains. Parasitic to moulds.

P. funiculosum, Thom. *Colonies:* (potato agar) green, spreading, floccose; hyphae form ropes; reverse reddish-purple, agar coloured. *Conidiophores:* 20–80μ, from trailing hyphae. *Penicilli:* 125–160μ, one or two alternate appressed branches, bearing verticillate branchlets and dense verticils of parallel sterigmata. *Sterigmata:* 2–3 × 10–14μ. *Conidia:* cylindrical, later elliptical, green, 2–3 × 3–4μ, chains break up easily.

P. purpurogenum, Stoll. *Colonies:* conidial areas green, the later hyphae being studded with yellow granules; reverse yellow-red. *Conidiophores:* 3–3·5 × 100–300μ. *Penicillus:* symmetrically biverticillate. *Sterigmata:* 2·5 × 10–12μ. *Conidia:* 2 × 3·5μ, walls finely granular, chains divergent or tangled.

7D. Penicillus biverticillate, but characteristic sterigmata or colourings absent: *Miscellania. P. avellaneum.* (See 7A above.)

ASPERGILLI. The *mycelium* is a mass of *hyphae* (filaments), from which grow *conidiophores* (stalks), showing no division into cells (i.e. are *unicellular*, or *unseptate*); and forming at the tip a *vesicle* (swelling) which is constant in size and shape, within certain limits, for any one species. *Sterigmata* (small stalks), unbranched, grow from the vesicle, and segment at the tips into chains of *conidiospores* (*conidia*) always unicellular, but varying in size, shape, and colour from species to species. The conidiophores usually show at the base a "foot cell" specialized from the hyphae.

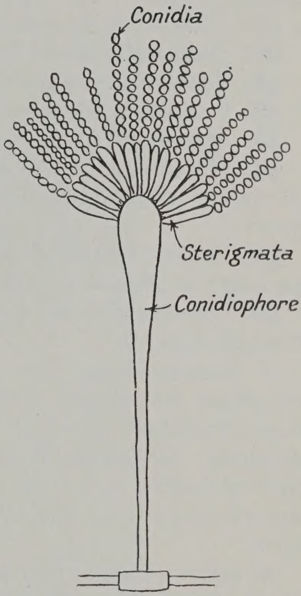

FIG. 192. ASPERGILLUS

Perhaps 10 per cent of the species also produce *perithecia*, which are closed fruit bodies, appearing as swellings on the mycelial hyphae. They are usually globular, fragile, thin walled, and yellow or orange in colour. In some cases they show other colours, such as black (*A. fumigatus*) or deep red (*A. herbariorum*). The perithecia are filled irregularly with *asci* (or bags), each containing eight *ascospores* (sometimes some other number), which are constant in size and shape in the species.

Key to the Aspergilli. The following key is adapted from Thom and Church, though only a few of the more commonly occurring type species are given in detail.

1A. Heads not clavate. (See 2.)

1B. Heads definitely cylindrical-clavate; vesicle cylindrical, not elliptical: *A. clavatus* group.

A. clavatus, Desmazières. *Colonies:* green, tipped blue-grey, rapid growing, densely matted, reverse and agar colourless, later brownish. *Mycelium:* hyphae 2–3μ diameter. *Conidiophores:* erect; walls smooth, colourless, thin; 15–20μ × 1–2μ. *Vesicles:* elongated, clavate, fertile area, 35 × 150μ. *Sterigmata:* unbranched, delicate, skittle-shaped, single series, crowded, 2–3 × 7–10μ. *Conidia:* oval, smooth, thin walled, almost hyaline, long chains, 2·5–3 × 3·5–4·5μ. *Heads:* 70–120 × 150–250μ. *Perethecia:* unknown.

2A. Stalk walls smooth, not pitted or rough. (See 3.)

2B. Stalk walls pitted, granular, rough. (See 11.)

3A. Conidial heads green or grey-green. (See 4.)

3B. Conidial heads never green. (See 7.)

4A. Sterigmata in one series only. (See 5.)

4B. Sterigmata in two series; heads contain both primary and secondary sterigmata (except in a few species), mostly green. (See 6.)

5A. Conidia elliptical or piriform, smooth or rough, more than 4μ long: *A. glaucus* group.

5B. Conodia globose, less than 4μ long, chains in columns: *A. fumigatus* group.

A. brunneofuscus, Sée. *Cultures:* rapid growing, black-brown, mycelium red-brown. *Conidiophores:* septate base, erect, sometimes branched, 10–15 × 150μ. *Vesicle:* ovoid or round, 35–40μ diameter. *Sterigmata:* unbranched, skittle-shaped, singly, on upper part of vesicle, 6–8 × 20–25μ. *Conidia:* round or ovoid, papillate surface, 8–15 × 12–18μ. *Head:* up to 75μ diameter. *Perithecia:* rare.

A. repens (Corda), Saccardo. *Colonies:* characteristic; dull green condiophores mixed with bright orange-brown perithecia; reverse uncoloured. *Conidiophores:* from aerial hyphae, smooth, thin walled, brown-green, 10–15μ diameter. *Vesicles:* very variable, 20–30μ diameter maximum. *Sterigmata:* 3·5–4·5 × 7–8μ; sometimes bear short conidiophore branches. *Conidia:* ovate, rough, 5–8μ long. *Heads:* radiate. *Perithecia:* small, orange-yellow. *Ascospores:* colourless, thick edged, no ridges, 3·7 × 4·7μ or slightly larger.

A. ruber (Spieckermann and Bremer), Thom and Church. *Colonies:* white, later greenish, later brown-red; reverse strong red. *Conidiophores:* from submerged or aerial hyphae, thin walled, smooth, 8–16 × 120–500μ. *Vesicles:* sub-globose-clavate, 24–30μ. *Sterigmata:* radiating, 2–4 × 6–10μ. *Conidia:* ovate, green, rough, 5–8μ. *Heads:* rather large, radiate. *Perithecia:* yellow brown, globose, 80–120μ. *Asci:* 11μ diameter. *Ascospores:* not ridged, show a smooth, shallow furrow, 4–5 × 6μ.

5B. Conidial globose, less than 4μ long, chains in columns: *A. fumigatus* group.

A. fumigatus, Fresenius. *Colonies:* green, later green-brown, reverse white-yellowish; velvety. *Mycelium:* hyphae, $2–3\mu$ diameter. *Conidiophores:* delicate, dwarfed, crowded, little differentiated from mycelium, grow from submerged or aerial hyphae, often green in upper part, $2–8 \times 300\mu$. *Vesicle:* flask-shaped, fertile on upper portion only, $10–20\mu$. *Sterigmata:* unbranched, crowded at summit of vesicle, erect, parallel to conidiophore, one series only, $2–3 \times 6–15\mu$. *Conidia:* smooth, round, or oval; dark green in mass, $2–3{\cdot}5\mu$ diameter, form long chains up to 400μ. *Head:* $40–100\mu$ diameter. *Perithecia:* brown, $250–350\mu$. *Asci:* oval, $9–14\mu$, 8 spores.

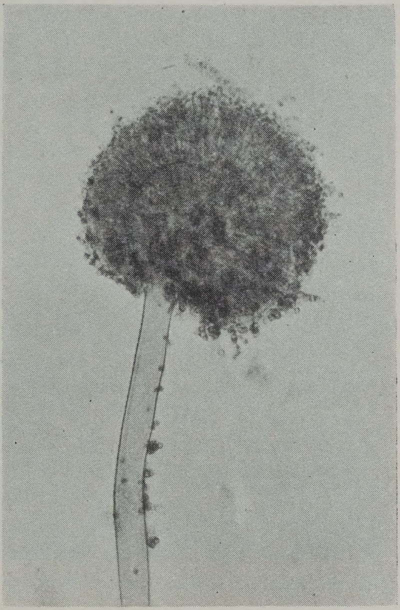

FIG. 193. ASPERGILLUS NIGER × 250 (*G. Smith*)

6A. Conodial chains columnar, stalks walls olive-brown or yellow-brown: *A. nidulans* group.

A. nidulans, Eidam. *Colonies:* white-yellow green, dark green; reverse and agar red-brown; velvety or floccose. *Mycelium:* orange-green, later dirty green, hyphae 6μ diameter. *Conidiophores:* smooth, often septate, branched, thick walled, mostly growing from aerial hyphae; hyaline, brownish when old, 3–5 × 50–100μ. *Vesicle:* little developed, flask-shaped, fertile on top half, 7–25μ diameter. *Sterigmata:* branched, slender, later swelling, hyaline; primary 2–3 × 5–8μ, secondary 2–2·5 × 7–10μ. *Conidia:* round, smooth, greenish, long chains often adhering to form large masses; 3–3·5μ. *Heads:* columnar, parallel chains, 40 × 100μ. *Perithecia:* when formed, are dark, slow ripening, thin walled, red-black, surrounded by thick walled (4–5μ) globular *Hülle cells,* 25μ diameter. *Asci:* reddish, 8-spored. *Ascospores:* violet-red, 4 × 5μ.

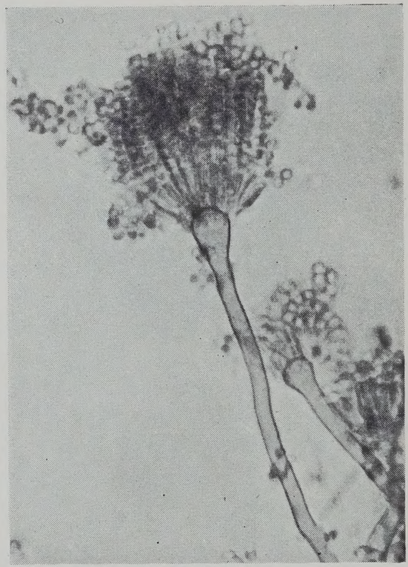

FIG. 194. ASPERGILLUS NIDULANS × 500 (*G. Smith*)

6B. Conidial chains not in columns, stalk walls practically colourless: *A. versicolor* group.

A. sydowi (Bainer and Sarbury). *Colonies:* deep blue-green, with white edges; reverse, red-brown or black. *Conidiophores:* thick, smooth walls, colourless, arise from submerged hyphae, 4–8 × 500μ. *Vesicle:* flask-shaped globose, fertile on upper half, 12–20μ diameter. *Sterigmata:* primary, 2–3·5 × 4·5–6μ secondary, 2 × 9μ. *Conidia:* globose, 2·5–3·5μ. *Heads:* small, radiate; small heads, common. *Chlamydospores:* on solid media undeveloped forms may occur, on submerged hyphae. *Perithecia:* unknown.

A. versicolor (Vuillemin), Tiraboschi. *Colonies:* pinky or greenish, reverse white or dull purple. *Conidiophores:* 3·5–4·5 × 350μ, walls

1μ. *Vesicles:* flask-shaped or globose, fertile all over or on upper two-thirds, 12–20μ diameter. *Sterigmata:* primary 2–3 × 5–7μ, secondary 1·75 × 5μ. *Conidia:* round, yellowish, almost smooth, change to jade-green when mounted in lactophenol, 2·5μ. *Heads:* 120μ maximum.

7A. Conidia smooth or spinulose, not pitted; no bars of colouring matter; not brown, purple, or black; thin walled. (See 8.)

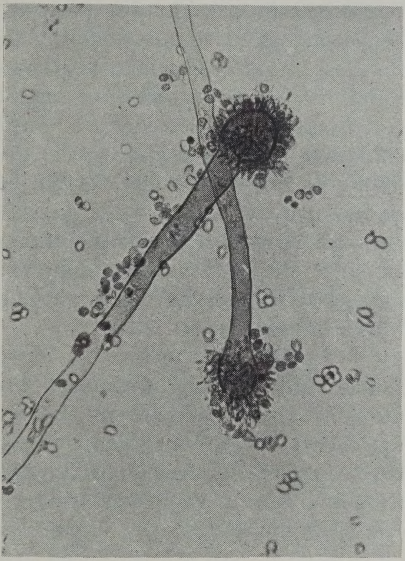

FIG. 195. ASPERGILLUS RUBER × 250 (*G. Smith*)

7B. Conidia roughened with bars of colouring matter; yellow, purple-brown, or black: *A. niger* group.

A. niger, van Tieghem. *Colonies:* very dark brown, rapid growing. *Mycelium:* submerged, hyphae 3μ, colourless. *Conidiophores:* smooth, shiny, slender, thick walled, hyaline, usually unseptate, yellowish in upper portion, 7–20μ × 0·2–2μ. *Vesicle:* often rough, cylindrical or globose, 80μ diameter, can only be examined after spores are removed. *Sterigmata:* branched, radiate uniformly from the whole surface of the vesicle, primary 3·5–7 × 40μ, secondary strongly coloured, 6–7 × 1·5–2μ. *Conidia:* round, dark coloured, when fully ripe is often warty and rough, 2·5–4μ. *Head:* white, later brown-black, 130μ diameter maximum.

7C. Conidia irregular, pitted; colonies orange-brown: *A. wentii* group.

A. wentii, Wehmer. *Colonies:* floccose, reverse yellow to red-brown. *Mycelium:* white, later red-brown. *Conidiophores:* long, slender, conspicuous, 10–15μ × 2·3 mm., wall smooth, colourless, thick. *Vesicle:* globose, covered all over with sterigmata; 75–90μ diameter. *Sterigmata:* vary greatly in size, primary 6–8 × 3–5μ, secondary 3 × 6–8μ. *Conidia:* round or elongated, roughened or pitted, 4–5μ. *Head:* white, later olive-brown, large; globose, later radiate, up to 500μ diameter. *Perithecia:* unknown.

8A. Conidial walls definitely spinulose. (See 9.)

8B. Conidial walls smooth, or almost smooth. (See 10.)

9A. Stalks colourless; heads columnar: *A. terreus* group.

A. terreus, Thom. *Colonies:* quick growing, velvety or floccose, colour sandy-cinnamon, reverse-yellow. *Conidiophores:* short, smooth; walls colourless, often septate; 5–8 × 50–150μ. *Vesicles:* characteristic, dome-like, upper surface fertile, 12–20μ diameter. *Sterigmata:* crowded, primary 2–2·5 × 7–9μ, secondary 2–2·5 × 5–7μ. *Conidia:* smooth, round; chains long, adherent; 2μ diameter. *Heads:* columnar, 50 × 500μ. *Perithecia:* unknown.

9B. Stalks fairly deep coloured; olive-grey; heads globose or hemispherical, brownish: *A. ustus* group.

10A. Outer layer of stalk walls yellow: *A. flavipes* group.

A. flavipes, Bainer and Sarbury. *Colonies:* white, glossy, later fawn; reverse brown. *Conidiophores:* yellow; buff, smooth, 4–5 × 300–500μ. *Vesicles:* elliptical or globose, 15–30μ. *Sterigmata:* colourless, crowded on apex of vesicle (small heads), or all over (large heads); primary 2–3 × 4–7μ, secondary 1·5–2 × 5–8μ. *Conidia:* smooth, round, colourless, 2–3μ. *Head:* usually columnar, white.

10B. Stalks have colourless walls (except at tips); heads white (or pale yellow when old): *A. candidus* group.

A. candidus, Link. *Colonies:* slow growing, white, later cream, reverse and agar colourless. *Conidiophores:* smooth, colourless, 5–9 × 500μ. *Vesicles:* globose, fertile all over, 4μ diameter maximum. *Sterigmata:* primary, colourless, 5–10μ diameter; secondary, 2·5 × 5–10μ. *Conidia:* round, colourless, hyaline, thin walled, 3μ diameter. *Head:* white, round or elongated, variable size, 200μ maximum.

11A. Colonies never green. (See 12.)

11B. Colonies green or yellow-green; walls marked with winding pits appearing echinulate under low powers. (See 13.)

12A. Outer layers of stalk walls yellow, often warty; heads yellow-ochre; sterigmata in two series: *A. ochraceus* group.

A. sulphureus, Desmazières. *Colonies:* light brown, later darker.

Mycelium: hyphae yellowish, 2·5–3·5μ *Conidiophores:* erect, often aggregated into coremia, not septate, hyaline, 4·5 × 230μ. *Vesicles:* inverted pear-shaped or rough, 15–30μ. *Sterigmata:* forked. *Conidia:* round, yellow to yellow-brown, smooth, 2·5–3·5μ; chains show no intermediate connections, long chains arise from all over the vesicle. *Head:* 50μ diameter.

A. ochraceus, Wilhelm. *Colonies:* ochre conidial masses. *Conidiophores:* thick walled, pitted; upper portion yellow; 7–10·5μ × some mm. *Vesicle:* globose, fertile all over, 40–75μ diameter. *Sterigmata:* primary, septate, 2·5 × 70μ; secondary, 2 × 10–12μ. *Conidia:* 3·5 × 5μ, almost smooth, almost colourless.

12B. Stalk walls not yellow; conidia rough; heads brown-umber: *A. tamarii* group.

A. tamarii, Kita. *Colonies:* rapid growing, colourless, later olive-brown; *hyphae* submerged. *Conidiophores:* moderately thick walled, grow from submerged hyphae; pitted, 10–20 × 1,000μ. *Vesicles:* thin walled, fragile, abruptly swollen, 25–30μ. *Sterigmata:* primary. 3–4 × 7–10μ; secondary, 3 × 7–10μ, or absent in small heads, *Conidia:* globose, strong inner wall, outer surface roughened by dark granules, 5–7·5μ. *Head:* variable size, globose or elongated, 350μ maximum.

13A. One series (occasionally double) of sterigmata, stalks long, heads yellow, almost no green tint: *A. oryzae* group.

A. oryzae, Ahlbury. *Colonies:* yellow-green to brown-green, later grey-green to dark brown. *Mycelium:* vegetative hyphae white to green, 3–9μ diameter. *Conidiophores:* closely matted, erect, hyaline, 10–30 × 300–2,000μ. *Vesicle:* round club-shaped, 50–80μ diameter. *Sterigmata:* evenly distributed or crowded at summit, radially, 4–5 × 10–20μ. *Conidia:* smooth or papillate, 6–7μ, chains break easily. *Perithecia:* unknown.

13B. Sterigmata. Both simple and double, green colour distinct: *A. Flavus* group.

A. effusus, Tiraboschi. *Colonies:* floccose, white, reverse yellow-pink; conidial masses dull green-yellow. *Mycelium:* fertile aerial hyphae are swollen into elongated foot cells, from each of which arises a conidiophore. *Conidiophores:* from aerial hyphae, 6 × 100μ, from submerged hyphae, 10·5–13·5 × 500μ. *Vesicles:* globose flask-shaped; fertile on upper two-thirds, 20μ diameter; fertile all over, 40μ diameter. *Sterigmata:* primary, 2·5–3 × 7·5–9; secondary, 2·5–9μ or absent. *Conidia:* round or ovoid, finely roughened (pitted), 5μ. *Head:* small columnar, or large, radiate.

A. Flavus, Link. *Colonies:* yellow-green, dark brown-green, matted, reverse white-pink. *Mycelium:* vegetative hyphae greyish.

Conidiophores: occasionally septate, stipe portion roughened by colourless granules; walls colourless, pitted; 7–10μ × 9·4–0·7μ. *Vesicle:* round club-shaped, 30–40μ diameter. *Sterigmata:* unbranched, crowded on summit of vesicle, 6 × 20μ. *Conidia:* round, smooth, 4–8μ diameter, chains break up easily. *Head:* 85μ diameter.

Mucoraceae. The *mycelium* is a mass of *hyphae* (filaments), which in many species are specialized into *stolons* (runners). These creep along the surface of the medium and at intervals drop "roots" into it.

Sporangiophores (stalks) arise out of the mycelium, and may be branched (*verticillate*) or unbranched. *Monomucors* show no branching; *Racemomucors* show rare or numerous branchings, in racemes or corymbs; whilst *Cymomucors* have the branching definite in sympodia.

At the tips of the sporangiophores, *sporangia* are formed, which consist of a spherical shell or membrane, inside which the protoplasmic contents gradually become *spores*. Cross-walls are formed first, then bulging occurs to columella. The specialized tip of the sporangiophore protrudes into the centre of the sporangia, this portion being known as the *columella*.

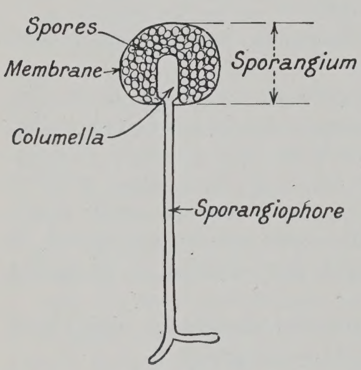

Fig. 196. Mucor

Under suitable conditions, two neighbouring filaments fuse or conjugate together, giving rise to sexual reproduction by the formation of black, warty swellings, known as *zygospores*. Zygospores are sometimes developed in the absence of sexual fusion. Filaments may expand into swellings, termed *suspensors*, near the spores.

Swellings, often black, thick-walled, intercalary—globose to barrel-shaped—also occur (on single filaments), which are similar to zygospores in appearance, termed *chlamydospores*.

When oxygen is not plentiful, especially in liquid media, the ends of the hyphae may abstrict off thin-walled *oidia* (yeast-like cells), capable of reproducing themselves by budding.

In the *Rhizopus* group, specialized aerial hyphae or *rhizoids* are produced, which attach themselves, e.g. to the lid of a petri dish, often very tenaciously.

Key to the Mucoraceae. The following key is after Lendner; the descriptions of species are based on Rabenhorst (*Kryptogamen Flora*) and Lafar (*Handbuch der technische Mykologie*)—

Monomucor.

1A. Sporangiophores at first erect, later drooping.

M. rufescens, Fischer. Forms a felt-like mass, rusty in colour.

1B. Always erect sporangiophores. (See 2.)

2A. Growth less than 300μ.

M. ramanniamus, Moller. On solid media, short velvety aerial mycelium, red-brown, later grey. *Sporangia* less than 20μ.

M. subtilissimus, Oudemans. *Aerial mycelium:* hardly visible. *Sporangiophores:* septate, colourless, 210μ. *Sporangia:* 40–50μ dia.

2B. Growth from 0·5–2·0 cm. high.

M. Hygrophilus, Oudemans. *Sporangial membrane:* not diffluent, breaking easily, leaving an irregularly torn collar. *Sporangia:* 36–42μ. *Spores:* elliptical, 6 × 8μ. *Aerial mycelium:* 1·5 high.

M. adventitius, Oudemans. *Sporangial membrane:* not diffluent. *Sporangia:* 80–100μ. *Spores:* elliptical, 5 × 8μ.

2C. Growth more than 2·0 cm. (See 3.)

3A. Oil globules present, mixed with spores.

M. plasmaticus, van Tieghem. *Sporangiophores:* erect, unbranched, or one or two side branches, 6–7 cm. high, colourless, smooth. *Sporangia:* round, 0·5–1·0μ; yellow, later yellow-green. *Sporangial membrane:* diffluent, no collar. *Columella:* oval or pear shaped, 160 × 250μ, colourless, smooth. *Spores:* oval, very unequal, 15–16 × 25–31μ, mixed with droplets of oil and granular protoplasm in sporangium.

3B. Oil globules absent. (See 4.)

4A. Growth 2–3 cm. high.

M. hiemalis, Wehmer. *Colonies:* silky, white or yellowish. *Hyphae:* irregular. *Sporangiophores:* erect, later collapsing, occasionally branched. *Sporangia:* globose, shiny, yellow-grey, 80μ. *Columella:* hyaline, globose-oval. *Spores:* thin walled, variable size, 8 × 10μ. *Zygospores:* not observed.

M. piriformis, Fischer. *Sporangiophores:* always erect, but inclined to droop, unbranched, or with sterile side branches, 35–50 × 2,000–3,000μ. *Sporangia:* white, later green-grey; outer wall spiny, membrane quickly diffluent, no collar, 250–350μ. *Columella:* large, smooth, pear-shaped, colourless; base, 80–110μ; top, 140–280μ; height, 200–300μ. *Spores:* uniform, ellipsoidal, smooth, colourless, 2–8 × 3–13μ.

4B. Growth more than 3 cm. high.

M. mucedo, Linn. *Sporangiophores:* unbranched, colourless, few contents, 30–40μ × 2–15 cm. *Sporangia:* large, round, yellow, later dark grey-brown; membrane dissolving rapidly, leaving collar. *Columella:* often yellowish, cylindrical or ovoid, 50–80 × 70–140μ.

Spores: rounded cylinders, various sizes, slightly yellowish, 3–6 × 6–12μ. *Zygospores:* spherical, 90–250μ. *Oidia:* not known. *Chlamydospores:* not known.

M. Mucillagineus, Brefeld. *Sporangiophores:* mostly unbranched. *Sporangia:* round, never yellow, but dark brown or black; membrane dissolving slowly. *Columella:* colourless. *Spores:* slightly yellowish contents, 15 × 30–33μ, embedded in a very slowly dissolving substance. *Zygospores:* unknown.

Racemomucor.

1A. Branching of sporangiophores verticillate.

M. glomerula (Bainer) Lindner. *Secondary branches:* verticillate, these again bearing verticillate branches.

1B. Branching of sporangiophores racemose or in corymbs. (See 2.)

2A. Columella hemispherical.

M. comatus, Bainer. *Columella:* covered with colourless hairs.

2B. Columella not hemispherical; round, oval, etc. (See 3.)

3A. Sporangiophores wilt and droop.

M. de Baryanus, Schostakowitsch. *Sporangiophores:* at first erect, later leaning over and drooping.

3B. Sporangiophores always erect. (See 4.)

4A. Not parasitic to mucors. (See 5.)

4B. Parasitic to mucors.

M. parasiticus, Bainer.

5A. Two kinds of sporangiophores.

M. agglomeratus, Schostakowitsch. *Sporangiophores:* (*a*) terminated by large sporangia, with diffluent membranes; (*b*) lateral, carrying sporangiola with persistent membranes.

5B. Sporangiophores of one kind only. (See 6.)

6A. One size only of spores. (See 7.)

6B. Two sizes of spores, one double the other.

M. heterosporus, Fischer. *Sporangiophores:* erect, 0·5–1·0 cm. *Sporangia:* 80–125μ diameter. *Spores:* round or angular, 4–15μ diameter.

M. sylvaticus, Hagem. *Sporangiophores:* 3–4μ. *Sporangia:* 70μ. *Spores:* oval or sub-cylindrical, 2·6 × 6–8μ. *Chlamydospores:* very large, along the sporangiophores.

M. lausanniensis, Lendner. *Sporangiophores:* 1 cm. *Sporangia:* 40–50μ, membrane fracturing.

7A. Sporangial membrane not diffluent, but rupturing into pieces. (See 8.)

7B. Sprangial membrane diffluent. (See 9.)

8A. Round spores.

M. corymbosus, Harz. *Sporangiophores:* 1–4μ, very much branched (mostly sterile). *Sporangia:* round, light yellow-brown, membrane not diffluent, tears irregularly. *Columella:* large, round. *Spores:* round, 7μ.

8B. Oval spores.

M. tenuis, Bainer. *Sporangiophores:* often not branched. *Oidia:* formed. *Chlamydospores:* covered with very fine prickles. *Azygospores:* numerous (Rabenhorst says: "zygospores in the form of azygospores").

M. racemosus, Fresenius. *Sporangiophores:* erect, yellow-brown, irregularly branched, all branchings bearing sporangia, 8–20 × 5–40μ. *Sporangium:* spherical, may droop, septum below; dull yellow-brown; membrane very strong, leaving collar after bursting; 20–70μ. *Columella:* egg-shaped, colourless, smooth; base, 7–30μ top, 9–42μ × 17–60μ high. *Spores:* irregular, round or elliptical, smooth, yellow, 5–8 × 6–10μ. *Zygospores:* round, brown, bearing yellow-brown warts, 70–80μ. *Suspensors:* much smaller than the zygospores. *Azygospores:* occasional. *Chlamydospores:* occasional, smooth walled. Colourless or yellow, occur in sporangiophore as well as mycelium.

9A. Round spores.

M. pusillus, Lindt. *Sporangiophores:* unbranched, later sparingly branched, and slightly bent; white, later brown; 10–20μ × 1μ. *Sporangia:* round, white, soon black; 60–80μ, membrane diffluent, leaving no collar. *Columella:* egg-shaped or round; smooth, pale yellow-brown, 50–60μ. *Spores:* round, smooth, colourless, 3–3·5μ.

9B. Oval or elongated spores. (See 10.)

10A. Very large species, 2-6 cm. high.

M. proliferous, Schostakowitsch. *Sporangiophores:* 6–7 cm. *Sporangia:* 300–400μ. *Spores:* 7·5 × 17·5μ.

M. flavus, Bainer. *Sporangiophores*, 6–8 cm. *Sporangia:* 140–160μ. *Spores:* 9–12 × 14·2μ.

10B. Species less than 2 cm. (See 11.)

11A. Columella largely subfacient to and concrescent with the sporangial membrane.

M. mollis, Bainer. *Sporangiophores:* erect, more than 1 cm., branched. *Sporangia:* round, 100μ, wall diffluent, smooth, no collar left. *Columella:* hemispherical, colourless, smooth. *Zygospores:* round, black, 80μ. *Chlamydospores, Oidia:* not known.

11B. Columella free, or slightly flattened at the base. (See 12.)

12A. Oval spores.

M. fragilis, Bainer. *Sporangiophores:* erect, 1 cm. maximum. *Sporangia:* small, round, black, membrane diffluent, smooth, leaving collar. *Columella:* round. *Spores:* Oval, smooth, blue-grey, 2·1 × 4·2μ. *Zygospores:* round, black, 50μ. *Oidia:* formed. *Chlamydospores:* unknown.

12B. Spores elongated, surfaces convex.

M. genevensis, Lendner. *Sporangia:* less than 80μ. *Zygospores:* frequent.

M. erectus, Bainer. *Sporangiophores:* erect, up to 1 cm. high, well branched, colourless, smooth. *Sporangia:* 80μ (50–120μ), pale yellow-green, membrane diffluent, leaving basal collar. *Columella:* round, 40μ. *Spores:* elliptical, smooth, colourless or grey; 2·5–5 × 5–10μ. *Zygospores:* round, 40–65μ. *Suspensors:* carry sporangiophores, as with *M. racemosus. Chlamydospores:* colourless. *Oidia:* unknown. *Azygospores:* numerous.

Cymomucor. 1A. Two kinds of sporangiophores.

M. pirelloides, Lindner. *Sporangiophores:* (*a*) straight, bearing normal round sporangia; (*b*) creeping, bearing piriform sporangia.

1B. Sporangiophores of one kind only. (See 2.)

2A. Curved sporangiophores. (See 3.)

2B. Straight sporangiophores. (See 4.)

3A. Species less than 1 cm. high; spores round.

M. circinelloides, van Tieghem. *Sporangiophores:* erect, branched (often regularly, left and right), 1 cm. *Sporangia:* often subsessile, round, brown. *Membrane:* not diffluent young, diffluent old. *Columella:* Hemispherical or round, colourless. *Spores:* smooth, round, or ovoid, 3 × 4–5μ. *Zygospores:* round. *Oidia:* formed. *Chlamydospores:* smooth, colourless.

M. griseocyanus, Hagem. *Sporangia:* carried on long curved pedicils; membrane blue-black. *Spores:* 4 × 5–6μ.

3B. Species more than 1 cm. high, spores oval.

M. augariensis, Schostakowitsch. *Sporangiophores:* spreading, 0·5–2 cm. *Sporangia:* black, 120–200μ. *Spores:* 10·5–14μ.

M. lamprosporus, Lendner. *Sporangiophores:* erect, not curved; but other shorter ones are present, much branched and curved. *Sporangia:* 60μ (90μ max.). *Spores:* 10–12μ.

4A. Spores unequal in size.

M. heterosporus sibericus, Schostakowitsch. *Spores:* round, unequal, shapes bizarre.

4B. Spores equal in size. (See 5.)

4C. Oval spores. (See 8.)

5A. Poor growth on grape juice gelatine.

M. Jansseni, Lendner. *Aerial myceium on bread:* 2–3 cm. high. *Sporangia:* 50–70μ. *Spores:* 5–6μ.

5B. Grow well on grape juice gelatine (aerial mycelium 2–3 cm. high). (See 6.)

6A. Spinous columella.

M. spinescens, Lendner. *Sporangiophores:* less than 2 cm. *Sporangia:* 60–68μ. *Spores:* smooth, 7–8μ.

M. Plumbeus, Bonorden. *Sporangiophores:* 1 cm. *Spores:* prickly, 5–8μ.

6B. Smooth columella. (See 7.)

7A. Pear or bell-shaped sporangia.

M. globosus, Fischer. *Sporangiophores:* wide branching, inclined to droop. *Sporangia:* round, grey-brown or dark brown when ripe, septum under each, collar remaining after resolution of membrane; 75–120μ. *Columella:* greyish, smooth, pear or bell-shaped, 6–8 × 40μ. *Spores:* blackish in mass, 4–8μ.

7B. Sporangia 110μ max., columella round, oval or bell-shaped, spores 10μ, sporangiola near the medium.

M. sphaerosporus, Hagem. *Sporangia:* 70–110μ; sporangiola not evanescent. *Spores:* 10μ, round, brilliant.

M. lamprosporus, Lendner. *Sporangia:* less than 80–90μ. *Sporangiophores:* higher than *M. sphaerosporus. Sporangiola:* curved, evanescent. *Spores:* 10μ.

M. dimorphosporus, Lendner. *Sporangiola:* absent. *Sporangia:* 60–80μ. *Spores:* (*a*) normal, round, 8–10μ; (*b*) oval, 8–10 × 30μ.

8A. Very large species, 9–12 cm. high.

M. irkutensis, Schostakowitsch. *Sporangiophores:* 9–10 cm. *Sporangia:* less than 1 mm. *Spores:* 10·5 × 28μ.

M. wasnessenskii, Schostakowitsch. *Sporangiophores:* 10–12 cm. *Sporangia:* 500μ. *Spores:* 5 × 8·6μ.

8B. Smaller species. (See 9.)

9A. Sporangial membrane not diffluent.

M. brevipes, Riess. *Sporangial membrane:* breaks into pieces.

9B. Membrane of first sporangia diffluent. (See 10.)

10A. Elongated prickly spores.

M. ambiguus, Vuillemin. *Sporangia:* black, 100μ.

10B. Subspherical, smooth spores. (See 11.)

11A. Poor growth.

M. rouxianus, Wehmer. *Colonies:* better on rice than on wort agar; slight yellow down only, on bread. *Sporangiophores:* erect or drooping, branched; short pedicils; orange-red matted mass on rice. *Sporangia:* cream, round, smooth, translucent, membrane transparent, collar after dissolution. *Columella:* free, colourless, smooth, slightly flattened. *Spores:* colourless, refractive, smooth, contents heterogeneous. *Chlamydospores:* numerous, variable in size and shape, 12–100μ diameter, colourless to yellow-brown; membrane colourless, smooth, thickened. *Zygospores:* not known. *Oidia:* formed.

11B. Growth 1–3 cm. high. (See 12.)

12A. Sporangiophores slightly branched.

M. geophilus, Oudemans. *Sporangia:* 50–350μ. *Columella:* round. *Spores:* round, angular, or elliptical, 4·2 × 5·6μ. *Chlamydospores:* formed.

M. strictus, Hagem. *Sporangia:* 90–170μ. *Columella:* ovoid. *Spores:* 5–6 × 6–8μ.

12B. Sporangiophores greatly branched.

M. prainii, Chodat and Nechitch. *Sporangia:* 35–70μ. *Spores:* 6 × 8μ or 8 × 10μ. Yellow pigment in hyphae.

M. javanicus, Wehmer. *Sporangia:* 50μ. *Spores:* oval, 4–5 × 5–7μ.

Rhizopus. **1A.** Spores round, not angular or oval. (See 2.)

B. Irregular, angular, subspherical, or oval. (See 3.)

2A. Spores spiny.

R. echinatus, similar to *R. nigricans*. *Sporangiophores:* unbranched, longer and thinner. *Sporangia:* round, small. *Spores:* round, grey-brown, 15μ. *Zygospores:* unknown. *Chlamydospores:* smooth, various shapes.

2B. Smooth spores.

R. elegans. *Stolons:* thick, long. *Rhizoids:* in bushels. *Sporangiophores:* seldom single; erect, branched with several short side arms, smooth, brown, 1–2μ high. *Sporangia:* round, small, end sporangia 50–70μ, side sporangia 33μ. *Spores:* round, smooth, light brown, 5–7μ.

R. speciosus. *Sporangia:* 90–140μ. *Spores:* 2–4μ.

3A. Smooth spores. (See 4.)

3B. Striate spores. (See 6.)

4A. Non-pathogenic.

R. chinensis. *Spores:* 5–7μ. *Sporangia:* 70–80μ.

4B. Pathogenic. (See 5.)

5A. Conical or sub-cylindrical columella.

R. niger.

5B. Ovoid or pear-shaped columella, pathogenic to rabbits.

R. cohnii. *Mycelium:* white, later grey. *Stolons:* not sharply differentiated. *Sporangiophores:* one or more, erect or bent, unbranched, smooth, brown, 120–125μ. *Sporangia:* white, later black; smooth, round, 60–110μ. *Columella:* smooth, brown, 50–75μ. *Spores:* round, 5–6μ; no blunt corners, smooth, colourless. *Zygospores:* unknown. *Chlamydospores:* unknown.

R. equinus. *Chlamydospores:* present.

6A. Sporangiophores bending over.

R. reflexus. Similar to *R. nigricans*. *Stolons:* up to 2 cm. *Rhizoids:* colourless, later brown, smooth. *Sporangiophores:* clusters, (4 or 5) unbranched, 2–2·5μ high. *Sporangia:* round, white; later black; 200μ. *Columella:* hemispherical, smooth, brown, often covered with fast adhering spores, 157μ. *Spores:* round or elongated, 8·4–10·5μ.

R. circinans. Sporangiophores: single, unbranched, 0·2 mm. *Sporangia:* round, black, small. *Columella:* brown, covered with spores. *Spores:* round-square, 5–6μ.

6B. Erect sporangiophores. (See 7.)

7A. Sporangiophores in groups. Spores more than 4μ. (See 8.)

7B. Sporangiophores singly, small; spores less than 4μ.

R. microsporus. Very similar to *R. nigricans. Sporangiophores:* single (or 2–3, occasionally), 0·4–0·8 mm. *Sporangia:* like *R. nigricans,* but one-third the size. *Spores:* 4 mm. *Stolons:* shorter than *R. nigricans. Rhizoids:* shorter than *R. nigricans.*

R. minimus. Similar to *R. nigricans,* but smaller. *Rhizoids:* 2–4 branches. *Sporangiophores:* 0·1–0·3 mm. *Sporangia:* one-tenth the size of *R. nigricans. Spores:* 3μ.

8A. Rhizoids well developed. (See 9.)

8B. Rhizoids poorly developed.

R. arrhizus. Rhizoids: rare, pale, short. *Sporangiophores:* no nodosities, 0·5–2·0 mm. *Sporangia:* round, white, later black, 120–250μ. *Columella:* 40–75 × 60–100μ. *Spores:* 4·8–5·6 × 4·8–7μ, oval or round.

R. nodosus. Rhizoids: rare. *Stolons:* branched. *Sporangiophores:* branched, swollen in places. *Spores:* oval, angular, irregularly polyhedric.

9A. Rhizoids irregularly distributed.

R. parasiticus. Rhizoids: irregularly distributed on stolons. *Sporangiophores:* branched. *Opt. temp.:* 37° C.

9B. Rhizoids normally distributed at the base of a sporangiophore cluster. (See 10.)

10A. No growth on potato at 39°C.

R. nigricans. Stolons: grow fast. *Rhizoids:* fairly well branched, colourless, later brown-black; thick, membrane smooth. *Sporangiophores:* seldom single, usually groups of 3–5; unbranched, smooth, brown-black, 24–42μ × 0·5–4 mm. *Sporangia:* hemispherical, white: later black, upright, 100–350μ. *Columella:* large, hemispherical. *Spores:* irregularly round or oval, usually one or two blunt corners; 6–17μ. *Zygospores:* round, 160–220μ, suspensors swollen, usually unequally.

10B. Growth on potato at 39° C.

R. tonkinensis. Fermentation: no action on sucrose, melibiose, raffinose, inulin; ferments trehalose.

R. japonicus. Fermentation: no action on trehalose; ferments sucrose, melibiose, raffinose, and inulin. Related species: *R. tritici, R. tamari, R. cambrodia*

10C. Grows well on potato at 39° C.

R. oryzae.

YEASTS. These unicellular fungi are placed in the ascus-producing group because under suitable conditions the cell itself becomes an ascus in which spores are formed in any number from 1–12, four or eight being common. The conditions for spore formation are young and vigorous cells, a temperature of about 25° C., and a suitable substratum. An excellent method is to make a block of plaster of Paris, which is slightly hollowed, and is made to stand in a little water in a Petri dish. The diluted yeast is streaked on the block and incubated for 24 hours without lid. The cell contents become granular and finally aggregate into spores, after which the parent cell swells and bursts, thus liberating the spores. The internal structures of yeast cells may be very complicated, including an inside wall, albumen, crystalloids, nuclius, oil globules, glycogen vacuoles, ordinary vacuoles, and protoplasm.

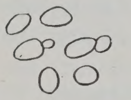
FIG. 197.
YEAST

The more usual and normal method of reproduction is by budding, a small protruberance forming on the cell, which splits off after a while and grows to the normal size for the species.

The Zygosaccharomyces reproduce by conjugation, a tubular beak being produced in place of a bud. When the beaks of two cells meet, union occurs, with subsequent ascospore formation.

When reproduction occurs by one of the above methods, the organism belongs to the Saccharomycetaceae. Reproduction may also occur by transverse division and the separation of daughter cells, as in the class Schizosaccharomycetaceae. An example is—

Schizosaccharomyces Pombe, Lindner. *Cells:* cylindrical, 4–9 × 5–9μ, one end rounded, the other end sharp or irregularly round. *Sporing:* easily takes place in hanging drop cultures, 1–4 spores being formed, 4μ diameter. *Fermentation:* dextrose, maltose, saccharose.

Fermentation of sugar is, of course, the characteristically important property of the yeasts, and is brought about by the presence of enzymes. Some yeasts contain invertase, which enables them to invert maltose and cane-sugar, in addition to being able to ferment the resulting hexoses to alcohol and carbon dioxide. The enzyme content of yeasts is very various, but no yeast is known which can ferment other than the hexoses directly.

The following enzymes occur frequently. *Endotrypase:* occurs in old yeasts, causing self-fermentation, albumen and glycogen producing alcohol, carbon dioxide, and soluble N compounds which escape out of the cell. *Lipase:* a fat splitting enzyme, producing glycerine. *Amylase:* which splits dextrin (occurs in *Schizos. Pombe*). *Inulase:* occurs in *S. marxianus* and *Schizos. Pombe. Invertase:* obtainable with difficulty except from old and dead cells; occurs in most yeasts (except many Torulas). *Maltase:* occurs in most yeasts, except the lactase-fermenting yeasts, in which it is replaced by lactase (e.g. in *s. Kefir*). *Melibiase:* occurs in bottom yeasts, but not in top yeasts. *Peroxidases:* these are almost always absent.

Comparatively few yeasts are industrially important. Cultivated yeasts are brewers' and distillers' yeasts, which are kept pure in strain, some cultures having been carried on for hundreds of years. Wild yeasts occur on the surface of fruits, e.g. wine yeasts; they may be beneficial, or produce bad tastes or smells, or they may have a negligible influence.

Yeasts may also be divided into the "top" and "bottom" yeasts. The former and more aerobic species grow chiefly on the surface, forming often a membrane, and ferment, e.g. raffinose to laevulose and melibiose; the anaerobic bottom yeasts can, in addition, split melibiose to dextrose and galactose.

Culture methods on solid media follow the usual lines. The most important media are wort agar, wort gelatine, and those media containing specific sugars. The plaster of Paris block is usually included for spore formation observation. It is also of considerable importance in identification to note the limits of temperature for spore and bud reproduction, and for skin formation on wort liquors.

The following two methods of obtaining *pure cultures* are widely employed.

LIQUID MEDIA. Half a gramme of pressed yeast is mixed with 10 c.c. sterile water, and one loopful is added to 10 c.c. water. A loopful is taken out and examined under a squared cover glass, on a squared slide, or by some other method which ensures the whole of the mount being systematically searched. The dilution is repeated until one loopful contains only one yeast cell. A loopful of this dilution is then added to each of a few Hansen flasks containing the medium, the chances being great that each will be a pure culture.

SOLID MEDIA (Lindner's method). Using the correctly diluted solution as obtained by method 1, a dozen drops are distributed by means of a sterile length of capillary tubing over a wort gelatine plate in a petri dish. Each drop probably contains only one yeast cell. Incubate the plate at 20° C. until growth takes place. Each spot in which growth has taken place is touched into a tube of liquid medium, which will probably be a pure culture.

The efficiency of yeasts is determined by the activity of fermentation, either the amount of alcohol or the amount of carbon dioxide being measured. The latter method is, of course, the easier. A Meissl ventilation valve, which allows gas to escape, but not water, is attached to a CO_2 flask; or a Schroeter's apparatus may be used. The flask is weighed before and after the experiment, the difference being CO_2. Concentrated sulphuric acid is suitable as the water-retaining medium in the valve, and Pasteur's fluid is a good medium for the fermentation. Incubation is conveniently carried out at 25° C.

ACTION ON SUGARS. A very quick and convenient method is due to Beijerinck. His medium contains all necessary salts, but no sugar. A well-innoculated tube is plated out and spotted with drops dextrose, laevulose, saccharose, lactose, maltose, dextrin, etc. Citric, malic, tartaric, and other acids may also be included. Growth occurs on the spots where a fermentable sugar is situated.

The following key gives a brief review of the yeasts proper; for the quasi-yeasts, such as the Torula, Lafar's Handbuch may be consulted; this work has a very pronounced bias towards the requirements of the fermentation industries throughout.

Saccharomycetaceae. 1. SPORE SHAPE. *A*. Round, hat, or lemon shaped. (See 2.)

B. Needle or spindle-shaped. (See 7.)

2. CELLS IN SUGAR MEDIA. *A*. Grow at the bottom, and only later or not at all on the surface, or form no membrane. (See 3.)

B. Form a membrane immediately growth commences. (See 6.)

3. SPORE MEMBRANE. *A*. Single. (See 4.)

B. Double: Saccharomycopsis.

S. guttulatus, Robin. *Cells:* elliptical, with stumpy ends; 2–4 × 6–16μ. *Budding range:* 3·5–37° C. *Spores:* long, oval, 1–4 per cell.

4. FUSION OF CELLS. *A*. Does not occur. (See 5.)

B. Takes place: Zygosaccharomyces.

Z. Barkerii, Saccardo and Sydowi. *Budding range:* (wort agar) 10–38° C.; no skin formed. *Spores:* easily formed, 13–38° C. *Ferments:* saccharose, dextrose, laevulose; no fermentation of maltose, lactose.

5. SPORE GERMINATION. *A*. By budding: Saccharomyces. (See 8.)

B. A promycelium is formed: Saccharomycodes.

S. Hansen. *Cells:* various in shape, chiefly lemon-shaped. *Budding range:* 1–38° C. *Spore formation:* on gypsum only, 2 to 30–32° C.

3–4μ. *Fermentation:* dextrose and saccharose, not fermented; maltose, fermented.

6. SPORE-SHAPED. *A.* Round, hemispherical, irregular or square: Pichia.

Pichia membranofaciens, Hansen. *Cells:* long, oval, with many vacuoles. *Budding range:* 0·5–36° C. *Wort gelatine colonies:* grey, rapidly liquefied. *Spores:* formed easily, round or hemispherical, range 2 to 30–33° C.

B. Hat- or lemon-shaped: Willia.

Willia anomala, Hansen. *Cells:* small, oval. *Budding range:* 1–38° C. *Spores:* hemispherical, 2–4μ, range 2 to 30–34° C.

7. SPORE SHAPES. *A.* Needle-shaped: Monospora.

B. Spindle-shaped or feathery, with long flagella: Nematospora.

8. (SACCHAROMYCES) FERMENTATION

TABLE VIII—SACCHAROMYCES

	Dextrose	Saccharose	Maltose	Lactose	Type Species (Descriptions follow the table)
A . .	+	+	+	–	S. cerevisiae S. Pastorianus
B . .	+	+	–	–	S. Marxianus
C . .	+	–	+	–	S. Rouxi
D . .	+	–	–	–	S. mali
E . .	–	–	–	+	S. fragilis
F . .	–	–	–	–	S. Hansenii

S. cerevisae, Hansen. *Bottom yeast cells:* large, round; top yeast cells 6–15μ, similar in shape, but with some variations. *Budding range:* 1–40° C. *Spores:* 1–4 per cell, 2·5–6μ; range on gypsum, 9–37° C. Only a small number of the commercially employed varieties have been described.

S. Pastorianus, Hansen. *Sporing range:* (on gypsum) 4 to 27·5–31·5° C. A bottom yeast giving unpleasant taste and smell to beer.

S. Marxianus, Hansen. Small oval- or egg-shaped cells, often forming colonies, sometimes showing a mycelium. *Budding range:* 0·5–47° C. *Spores:* (gypsum) round or oval, 3–5μ, 4 to 25–34° C. *Skin:* after two or three months shows distorted shapes. *Solid media:* mycelium formed.

S. Rouxi, Boutroux. Round or oval cells, chains formed, size very regular, 4–5μ. *Skin:* none formed.

S. mali Duclaux, Kaiser. *Cells:* 4–8 × 6–12μ. *Skin:* formed. *Spores:* after 84 hours at 15° C. A top yeast in cider.

S. fragilis, Jorgensen. Small oval cells, spores elongated and round.

S. Hansenii, Zopf. Cells round or ellipsoidal, 4–11μ, contain oil globules, gelatine not liquefied. *Spores:* 2–4μ, 1–2 per cell. Oxalic acid formed in dextrose, lactose, maltose, and saccharose.

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CHAPTER XVI

WATER AND SEWAGE

Collecting of Water Samples. A 2-oz. bottle is usually sufficient for a bacteriological examination. The bottles are sterilized with the stopper inserted loosely, and preferably in the container in which it is to be carried. A cocoa tin is very convenient for this purpose. When the sample is being taken great care should be exercised that the stopper touches nothing but the water. The bottle should always be opened a little below the surface in order to avoid contamination. A good deal of water should be pumped away from wells before taking a sample. When water is to be collected from various depths a string may be tied to the stopper, which can then be pulled out at the correct depth.

The following points should be recorded: previous rainfall; source —river, well, etc.; depth of sample: details of piping, outflow point, etc.; day and date.

A complete analysis of water is only undertaken when it is desired to ascertain its suitability for any particular purposes, and this analysis must be supplemented by some general information about the source of supply. In many cases this may be obtained from the analytical data with some degree of accuracy, the chief data of value in this connection being the microscopical examination of the sediment or deposit from the sample. In many cases the indications obtained are almost equivalent to having actually visited the source. For example, the presence of clay or sand will indicate running water, or humus colouring matter will point to a moorland source. Stagnant water is indicated by the presence of organisms such as amoebae and vorticellae, whilst filamentaceous algae would betoken a still pool. Planarian worms are often found in spring waters. Fibres from cloth would point to a well near a house, and so on *ad infinitum*. It is scarcely possible here to indicate more than the lines of argument followed, based upon the data given below for typical examples. In practice, of course, other organisms may occur, or some of the life may be missing. Thus a deep well which allows access to surface water would contain, in addition to a little clay and sand, organisms such as amoebae and rotifers; if unprotected from dirt, one may expect dead insects, bits of leaves, etc.; and if the surface water came from the moors humus colouring matter would be present.

RAIN-WATER. *Deposit.* Black amorphous soot, traces of sand, a few green algae and spores. *Bacteria.* Few bacteria and no excretal organisms.

UPLAND SURFACE WATER. *Deposit.* Diatoms and sponge spicules; rotifers; desmids, a little sand; if from peaty moors, some humus colouring matter (brown amorphous particles not decolourized by HCl); leaves of moss from bogs. *Bacteria.* Many, but no Esch. coli from uncultivated soil. From moorland soil excretal organisms from grazing animals and wild-fowl may occur, hence Esch. coli is often present in large numbers, even when there is no possibility of contamination from human sources. Animal excreta is much less dangerous than human. In general, the presence of Esch. coli in less than 1 c.c. is bad. If less numerous, the ground should be examined before passing an opinion.

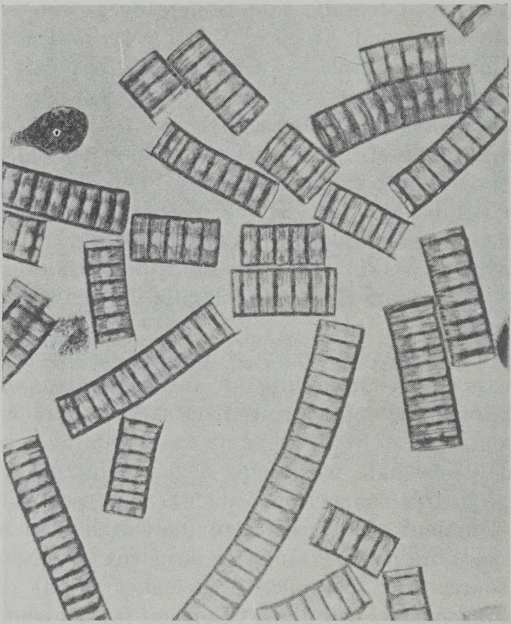


FIG. 198. ORTHOSIRA GRENARIA

SPRINGS. *Deposit.* The characteristic features are sand and planarian worms. The chemical analysis is similar to that of water from deep wells.

DEEP WELLS. *Deposit.* Chiefly sand-clay and amorphous matter coloured brown by iron (colour discharged by HCl). *Chemical analysis.* As the shaft usually passes through impervious strata, the water will have been well filtered and oxidized during percolation (provided surface water is excluded); and hence the past history of the water is of less importance, so far as contamination at the source is concerned, than the present condition. *Bacteria.* Due to filtration through the deep layers of soil, there are few bacteria and no Esch. coli. Hence the presence of Esch. coli indicates pollution with surface water. When Esch. coli is present in 100 c.c. or less, the water is to be regarded with suspicion. It should be noted, however, that well water is often contaminated after leaving the well-head.

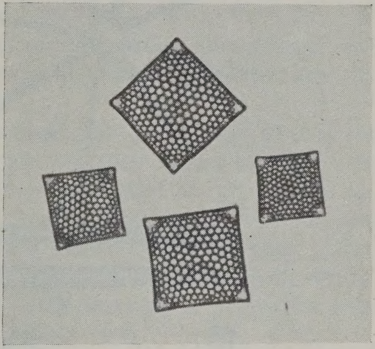


FIG. 199. TRICERATUM SP.

RIVER WATER. *Deposit.* Much sand and clay, diatoms, particles of decaying vegetable matter, nematode worms, rotifers. *Chemical analysis.* Varies greatly with rainfall, sewage contamination, and seasons. *Bacteria.* Usually high, especially when draining cultivated land. No standards are possible.

STAGNANT WATER. *Deposit.* Many amoebae and vorticellae (characteristic of still waters), waterfleas, larvae of aquatic insects, rhizopods (swampy ground), green filamentous algea (grassland pool), diatoms, brownish dirt. *Bacteria.* Many large bacteria, but very wide variations are found. When the entrance is protected from surface water (wells) and filtered through 12 ft. of soil, there are usually none in 50 c.c. and certainly none in 10 c.c. Wells with entrances through the mouth and sides are very common, and usually show heavy pollution. This applies also to wells with less than 12 ft. of filtration. In general, bacteria should be absent from 10 c.c. of surface water. *Chemical analysis.* Shallow wells behave as if they were the dumping ground for the drainage of the surrounding soil, and as they are almost always near houses they contain the soluble products derived from the excretal, etc., refuse.

SEWAGE DEPOSIT. White flakes of filaments of cladothrix, dichotoma, Beggiatoa, large bacteria, vorticelli, paramoceia, euglenae.

GENERAL NOTES. Other deposits, such as sawdust, fragments of feathers, and cloth fibres, are usual from household dust. Traces of starch grains occur in wells in farming districts, to which surface water has had access, but large quantities indicate contamination by household waste liquor, when skin flakes will also be found. Nematode worms occur in most waters. Iron pipes give crenothrix filaments and particles of iron rust. Near-by trees cause spores of septoria from decaying leaves to occur. Cretaceous formanifera indicate that the water has passed through a chalky district.

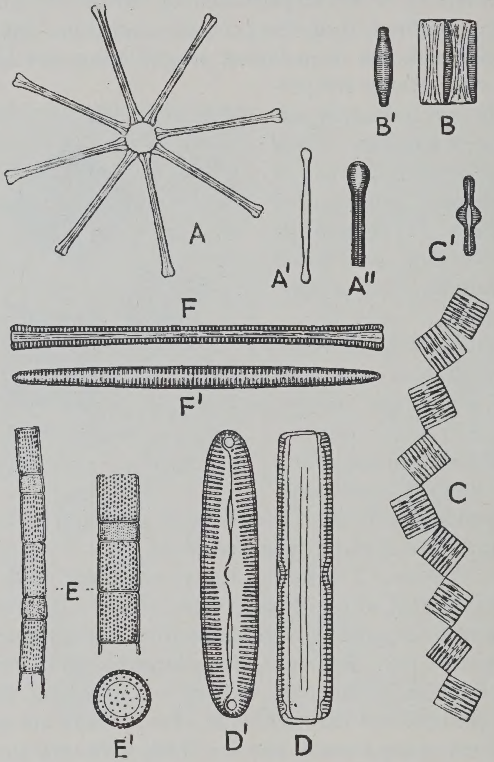


Fig. 200. Diatoms Common in Reservoirs

(All magnified from 300 to 500 diameters.)

A, A', A'' = *Asterionella formosa.*
A = Eight specimens joined in a stellate group.
B, B' = *Fragilaria virescens.*
C, C' = *Tabellaria flocculosa.*
D, D' = *Navicula viridis.*
E, E' = *Melosira granulata.*
F, F' = *Synedra ulna.*

Figs. *A, B, C, D, E, F,* Girdle view; the remaining figures, valve view.

Bacterial Contamination of Water. In general, the number of bacteria is increased by soil washings, drainages of cultivated and uncultivated land, or from human habitations, and by high organic content. The number is decreased by sedimentation, deficiency of food, and the action of light, especially of sunlight. The greater part of these bacteria are non-pathogenic, and in the examination of water for its suitability for drinking search is normally made for the so-called indicator organism—Esch. coli.

The reasons for this are that this organism is abundant in the intestinal tract of man, animals, and even fish and birds, but does not multiply as a rule very much outside the animal body; it is relatively absent from all other sources; it is fairly easily isolated and estimated numerically, and its characters are fairly definite and not subject to great variation.

When Esch. coli is found on soil or water, it is, therefore, a sure indication of comparatively recent contamination by manure, excreta, or sewage.

SEARCH FOR ESCH. COLI. McConkey's lactose bile salt agar is the most valuable medium. The growth of other organisms likely to occur is considerably retarded when incubated at 37° C.; 10 c.c. of the medium is placed in each of three sterile tubes; 0·1, 1·0, and 10 c.c. of water is added to the three tubes, which are then plated out and incubated at 37° C. for two days. All roundish, white, irregular colonies, with flattened tops, showing a yellow or gold spot in the centre, may be taken as Esch. coli.

COUNT OF ESCH. COLI. The usual methods are adopted, as given in Chap. XIII. Plates should be made on both gelatine and agar, and counts taken every 24 hours, the count on the third day being stated.

IDENTIFICATION. The following cultures are advisable: agar streak gelatine stab, gelatine plate, milk, lactose broth, glucose broth; each colony found on the McConkey agar should be subcultured by this method, though only three need be worked out fully. The distinguishing characteristics of Esch. coli, and related organisms which serve to separate them from the Proteus, Gartner, Dysentery, etc., groups, are given by Savage as follows: Small non-sporing bacteria, gram negative, grow well at 20 and 37° C., ferment glucose and lactose giving acid and gas, motile under suitable conditions. The following more detailed tests may be added: indol is produced in peptone water; growth on gelatine streak is translucent and non-corrugated; gelatine is not liquefied after two weeks; acid is produced in litmus milk, which is coagulated after two weeks; greenish-yellow fluorescence is shown in glucose neutral red broth; aesculin-ferric citrate agar is blackened. There are many bacteria which conform more or less closely to this specification, and care should be taken against a too strict insistance on all the data mentioned.

Examination of water for specifically pathogenic organisms should only be undertaken by a worker who has received training at the hands of an experienced bacteriologist in bacteriological technique.

It should be remembered that bacteria supply information about present pollution only, not about possible pollution, which may be intermittent. For this reason, water containing Esch. coli in less than 100 c.c. should be strongly condemned, whilst caution should be exercised if it is absent. An examination for *B. enteritidis* is often helpful, for this organism, being spore-bearing, is much more resistant than Esch. coli, and its absence is a strong indication of purity. Even in soil this organism is known to have survived for a considerable time after the original pollution, whereas Esch. coli soon dies out.

Non-sporing bacteria should be killed by heating to 80° C., and an anaerobic culture made in nutrient agar, 5–7 days at 37° C. Four tubes, each containing 15 c.c. of milk, are then sterilized by steam for half an hour, which expels carbon dioxide. They are then rapidly cooled in cold water, inoculated with suitable dilutions, and incubated at 37° C. for 1–2 days. The so-called "enteritidis change," which can also be caused by *B. butyricus* or *B. cadaveris*, though neither are probable in sewage, consists of the formation of an acid whey, a smell of butyric acid, a surface coating of pinky-white coagulated casein which encloses gas bubbles, the cream being torn in a rather characteristic manner by the gas, which is liberated on shaking.

B. enteritidis may be present in very small quantities only, even in badly polluted water, hence the test should be carried out with 10, 100, 500, and 1,000 c.c. of water, which are concentrated by evaporation.

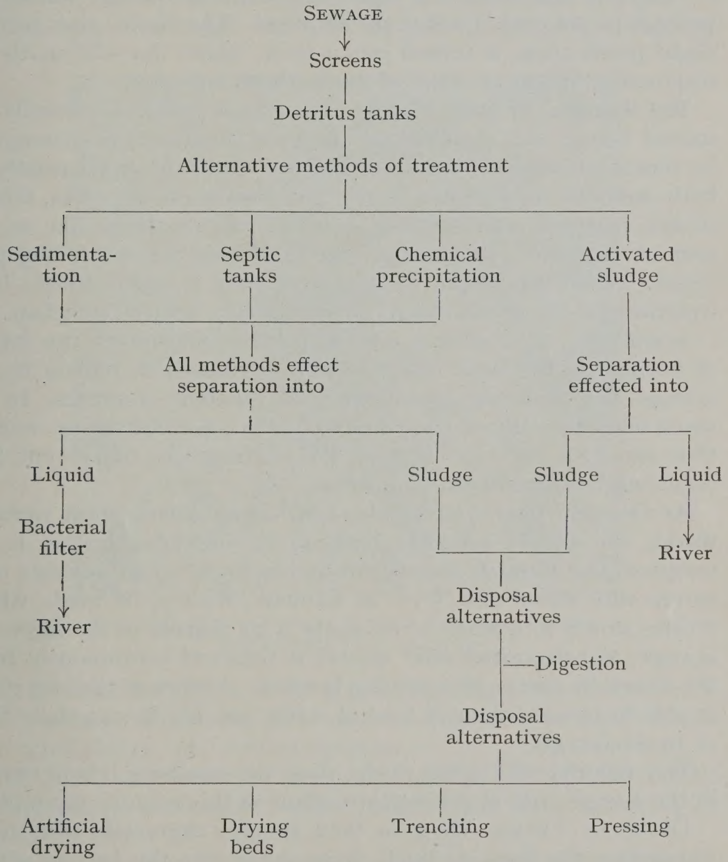
SEWAGE. Only sixty years ago all waste waters were turned into the nearest stream as a matter of course, and it was not until 1865 that a Royal Commission was formed which eventually brought about the "Rivers Pollution Act" of 1876.

Since that date the ever-growing population of the country, and the birth of new industries, have resulted in the producer of a putrescible effluent being compelled either to treat it before it leaves the works or to turn it into the local sewers and pay for its treatment by the local authorities.

Sewage is a dirty grey liquid possessing an unpleasant foetid odour; contained in it are fruit skins, vegetable waste, faeces, corks, and other matter from household waste, in addition to all or nearly all of the various chemicals, both organic and inorganic, which are used in manufacturing processes in the district. The bacterial content is very high, usually more than 1,000,000 per c.c., and often as great as 100,000,000.

The object of sewage treatment is to produce a final effluent which is non-putrescible and free from pathogenic organisms; when correctly processed it becomes a bright, sparkling liquid. The methods of accomplishing this object are very various; they are briefly summarized in general outline by the following diagram—

DIAGRAMMATIC REPRESENTATION OF SEWAGE TREATMENT


SEWAGE
Screens
Detritus tanks
Alternative methods of treatment
Sedimentation
Septic tanks
Chemical precipitation
Activated sludge
All methods effect separation into
Separation effected into
Liquid
Sludge
Sludge
Liquid
Bacterial filter
River
River
Disposal alternatives
Digestion
Disposal alternatives
Artificial drying
Drying beds
Trenching
Pressing

In the case of weak sewages, simple screening is adopted to remove heavy bodies, the effluent being then allowed to drain through the soil or through the artificial equivalent, bacterial filters, the purification in both cases being effected by the soil bacteria.

When, however, trade waste liquors come into consideration with consequent high concentration of solids, an intermediate treatment is necessary, which may take one of four forms, namely, *sedimentation,* which is a settling process; *chemical precipitation,* in which the purification is effected by the addition of lime, acids, etc.; *septic tank,* which is a biological method; or *activated sludge,* which also purifies by bacterial means. The biological processes have as their object the breaking down and consequent removal of solid matter.

Whatever the method, a liquid effluent, and a sludge containing perhaps 90 per cent of water are obtained. The liquid, after further slight purification, is turned into a river, whilst the solid matter is disposed of by one or other of the methods indicated.

The Removal of Solid Matter. The grosser solids are usually removed before any chemical or biological treatment is attempted, by means of either screening or detritus tanks, or more usually by both methods in sequence; sand, grit, cotton waste, corks, faeces, stones, matches, and the like, which form something like 25 per cent of the total solid matter, merely impede the succeeding processes. When the proportion is 50 per cent or more, simple land treatment of the effluent from screening, etc., is often sufficient.

SCREENING. The screens used in practice depend on the degree of fineness of the solid matter which is allowed to remain in the sewage, this differing considerably in different countries. In all cases, much difficulty is encountered by the screen becoming choked, thus arresting the rate of flow, the contrivances to prevent this occurring being many and ingenious.

At Dresden, for example, where, in common with most German works, the almost complete removal of suspended matter is attempted, the Reinsch installation is employed. This consists of a woven wire screen disc, 5 yd. in diameter, with $\frac{1}{12}$-in. mesh, which rotates slowly in a plane at an angle of 23 degrees to the incoming sewage; the deposited solid matter is removed continuously from the screen by means of revolving brushes. A sieve of the size given is able to remove about 8 tons of solids per day from a daily flow of 10,000,000 gal.

The majority of English works allow the smaller solids to remain in the sewage, and in consequence plant in this country is simpler.

DETRITUS TANKS. These in their simplest form take the shape of cisterns, the floor of which slopes down into the bottom of the inlet end. The heavy matter settles down into the bottom of the tank, and is removed at regular intervals, either by hand or by a steam-operated grab. The size and number of the tanks is usually such that the flow of sewage is not more than 2 in. per second, and the total tank capacity is one-hundredth of the average dry weather flow. An auxiliary set is necessary in order to facilitate cleaning.

The principle has been much elaborated in certain works; for example, in the Schneppendahl system a series of perforated buckets is employed, which are lowered into the tank and removed full of detritus by means of a crane.

Biological Processes. When, as is usually the case, the foregoing methods alone are inadequate, biological processes are usually employed. These may be divided into *processes which take place in the atmosphere,* amongst which are irrigation, sewage farming, and such artificial developments as percolating filters and contact beds; and *processes which take place under water,* such as septic tanks, activated sludge, and contact aerators.

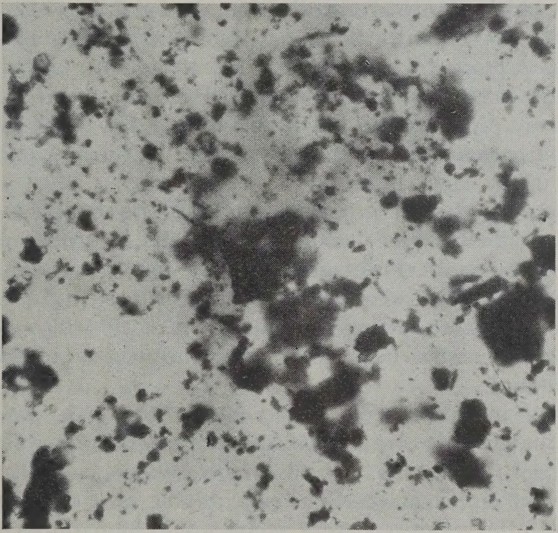


FIG. 201. SLUDGE FROM SIMPLE SEDIMENTATION—NEGLIGIBLE COAGULATION

All these methods seek to control and accelerate the normal purification of putrescible matter, which takes place in nature by the action of the soil bacteria.

IRRIGATION. Until the introduction of modern methods, irrigation on land was the only available method of disposing of sewage, and this process is still practised in villages where the sewage is comparatively weak.

The action may be regarded as filtration through land, the bacteria of the soil acting as purifying agents; and, in consequence, impervious soils, such as peat and clay, are unsuitable, though a good loam will produce a bright stable effluent fit for any stream, provided attention be paid to the distribution of the sewage over the surface, and the prevention of short-circuiting. The two chief methods are described below.

RIDGE IRRIGATION. This consists of running the sewage into furrows constructed on the apex of a long ridge, the sewage percolating through the soil, or, when the furrow is full, overflowing down the long ridge, and so passing into the soil.

BED IRRIGATION. The sewage is run into channels cut between beds on which plants are grown, but does not flood the soil, and comes into contact with the roots only of the vegetation. A variant of this is *subsoil irrigation,* which is occasionally employed when the sewage must not be on the surface at all; and in this method earthenware pipes are run a foot below the surface, the joints being loose, and packed round with stones and like substances to prevent choking up by earth and roots.

Revenue may be obtained from such irrigation methods by means of the so-called sewage farming. It has been estimated that the annual commercial value of a sewage (as a manure) is equal to 4s. per head of population. Grass and root crops, such as turnips, are chiefly grown, but it is interesting to note that recently willow for cricket bat manufacture has been very successfully cultivated on sewage-manured land.

Although irrigation is cheap and efficient for weak sewage, it fails entirely for effluents containing large amounts of trade waste flushes; whilst when the daily flow is millions of gallons, as is the case with large towns, sufficient land is not available near the towns, quite apart from the enormous areas which would be required.

For this reason, artificial irrigation grounds, or biological filters, have been devised, which, in contrast to the naturally formed soil filter, are uniform, and will deal with much greater quantities of sewage. They are breeding grounds for the bacteria natural to the soil, and they serve to remove all traces of putrescible matter dissolved in sewage which has been screened, or from the liquid effluent from one of the sewage treatments to be described later.

BACTERIA BEDS. These beds consist of small pieces about the size of a walnut, of coal, stone, shale, clinker, or any suitable material which will act as a breeding surface for bacteria, and will not disintegrate. The depth of the beds is one of personal choice, but no benefit is gained beyond 6 ft., and it is established that the bulk of the purification takes place in the first foot, or perhaps 2 ft.

The methods of mechanical distribution of the sewage over the beds vary considerably, and there is not a great deal to choose between half a dozen well-known ways, which provide uniform distribution over a maximum area. In all cases, when operating a new bed, the effluent for a considerable time is little better than the incoming sewage, but gradually a reduction of the dissolved solids occurs; and when 65 to 70 per cent of this organic matter is retained by the filter a non-putrescible effluent results. At this point each lump of the bed material is covered with a thin gelatinous film, which grows still thicker on further treatment. The film contains large numbers of bacteria and low forms of animal life, together with a quantity of iron salts. The structure approximates to a honeycomb, giving it an internal as well as an external surface; the solid matter is absorbed by the honeycomb structure, and certain substances, such as sugar and urea, are retained inside the cells by the action of osmotic forces.

The process of developing the film takes some weeks, and is termed ripening or maturing.

It is assumed that the purification is carried out in two stages, the peptone-like bodies, which are decomposition products of the albumen content of the sewage, being first absorbed by the film, and subsequently oxidized by the bacteria. Carbon-containing bodies are converted to CO_2, and nitrogenous substances are transformed into nitrogen, ammonia, and nitric acid as a final product.

This oxidation cannot be attributed to any single organism, for of the common forms found in filters *B. furfuris* attacks starch and sugar, but not cellulose; *B. subtilis* decomposes grass, yielding CO_2 and N_2; *Strep. longus* decomposes fibrin into ammonia, methylamine, trimethylamine, etc.; *B. mycoides* acts upon carbohydrates, and will hydrolize cane-sugar and maltose.

Certain of the lower forms of animal life also play a part. *Achorutes viaticus*, a wingless insect, is often cultivated on a clogged filter, in order to restore it to working order. Two species of *Psychoda*, *P. phalaenoides* and *P. sespunctata*, are also prevalent, but it is only in the larval stage that these serve any useful purpose; afterwards they are pests. It has been found that *Podura* are enemies of the *Psychoda*, and if cultivated will keep beds free from them in the fly stage.

A sewage containing a large amount of dissolved matter must undergo some special treatment, which may take the form of a biological digestion, either by aerobic or anaerobic organisms. Septic tanks and variants are purely an intermediate process producing a thick, black sludge which is not difficult to air-dry, and an effluent which after filtering on bacteria beds is fit to be turned into a stream.

The activated sludge method, however, which uses aerobic bacteria, is a complete process in itself, producing a brown flocculent sludge which holds water tenaciously (and is consequently difficult to dry) and an effluent which may be turned into a river without filtration through bacteria beds. It is on paper a much better method than the septic tank, but provides many practical difficulties.

Septic Tanks. These are really a modification of simple sedimentation tanks, the sludge which settles being allowed to decompose by a self-induced biological action; they are based upon an experiment by Louis Mouras, who constructed a cesspool which had the inlet and outlet always submerged.

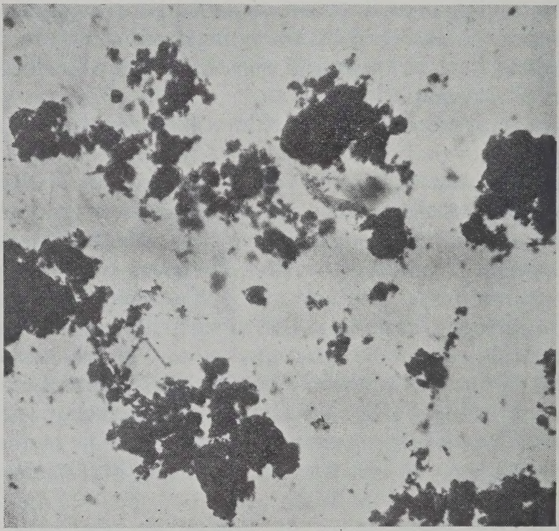


Fig. 202. Sludge from Septic Tank, showing Large and Probably Indigestible Aggregates

The sewage is allowed to pass slowly over the "septic" sludge, the time of contact depending upon the strength of the sewage treated. A considerable amount of the sludge in the sewage is "digested" and removed; pathogenic organisms are largely destroyed; and, incidentally, a considerable quantity of combustible gas is evolved. Unfortunately, sulphuretted hydrogen is present in the effluent, which is liable to cause harm to stream life, in addition to which the concrete tanks usually employed are often considerably corroded by by-products of the septic action.

Theoretically, closed tanks are necessary, but in practice open tanks soon make for themselves a natural seal, owing to the escaping gases carrying to the surface paper, hair, fat, and other matter; the scum thus formed readily grows moulds, thus producing a very tenacious coating. The scum contains a great deal of life, amongst which are worms (*Lumbricidae*), flies (*Psychoda*), and moulds, such as *Pilobolus oedipus*, which has brown spores, and *Peziza omphalodes*, which is red, and is often found on dung heaps.

Various striking experiments have been performed to demonstrate the action of these tanks. Large bodies, such as cabbages, potatoes, skinned and unskinned animals, and bones, suspended in the tank are almost completely dissolved in less than a month, whilst a skinned guinea-pig was cleaned to the bones in less than three weeks.

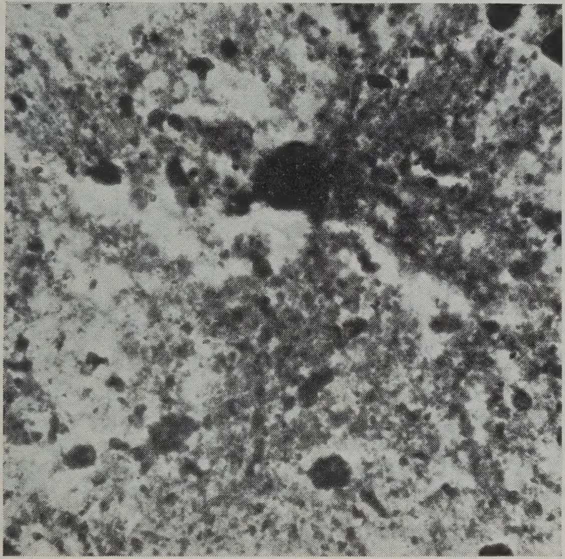


FIG. 203. SLUDGE FROM LIME PRECIPITATION—TRANSLUCENT PARTICLES (HYDROXIDES) AND SMALL AGGREGATIONS

Opinions differ greatly as to the amount of purification obtained in a septic tank, but it is safe to say that septic tanks reduce the dissolved solids by at least the same amount as does chemical precipitation. The effluent is not, however, pure enough to be turned into a stream, and must be filtered on bacteria beds. This is much more simple than the filtration of ordinary settled sewage, because roughly 60 per cent of the suspended solids and 50 per cent of the dissolved solids have been removed.

A debated question is whether or not the remaining organic matter, which is, of course, retained by the filter, is easier than the untreated to decompose by the filter. It is certain that sewages containing large quantities of carbohydrates, e.g. effluents from sugar factories and breweries, are not responsive to septic tank treatment, because they develop acid, which acts as a germicide to the bacterial life.

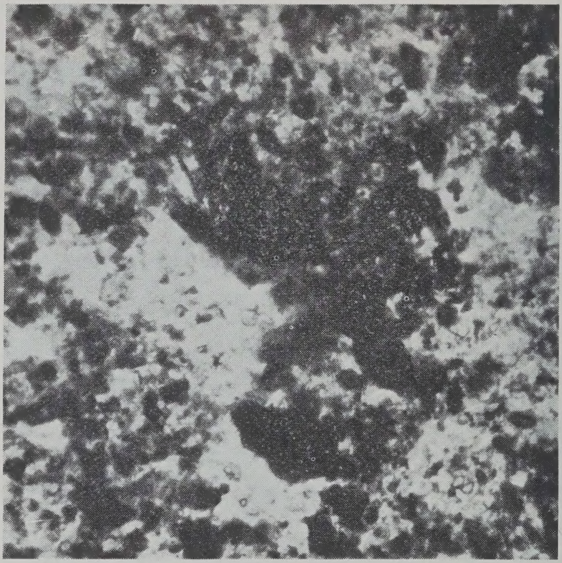


Fig. 204. Sludge from Acid Precipitation—Large, Coarse Aggregates

In all septic tank installations, where the incoming sewage is subject to strong influxes of trade wastes, it is advisable to average these by passing the whole through a tank fitted with baffles.

Activated Sludge. The modern tendency is towards the intensification of the process, and the activated sludge treatment is an outcome of this line of thought. Boswell describes it thus; "Activated sludge flocks are composed of a synthetic gelatinous matrix, similar to that of vestros or microsmopedia, in which filamentous and unicellular bacteria are embedded, and on which various protozoa and some metazoa crawl and feed. The purification is accomplished by digestion and assimilation by organisms of the organic matter in the sewage and its re-synthesis into the living matter of the flock. This process changes organic matter from colloidal and dissolved states of dispersion into a state in which it will settle out."

In brief, activated sludge is an artificially accelerated self-purification, effected by harnessing the natural purifying agencies of the rivers and the earth.

As, however, a large number of organisms are concentrated into the relatively small space of an activated sludge tank, artificial aeration, to supply oxygen, must be accomplished. The sewage passing through the tank must be kept agitated to prevent settlement of the sludge, as any sediment would quickly become septic. The sludge, in fact, is often settled before entering the activated tank, in order that the process may only treat the dissolved matter. In addition, industrial effluents of a strongly alkaline or acid character must be well distributed in the sewage, in order to prevent any detrimental action on the activated sludge.

The mixing and aeration may be accomplished in various ways, these now to be described being amongst the most important.

COMPRESSED AIR METHOD. In the early type of plant, perforated pipes were employed, but these proved wasteful of air, and easily choked. They are at the present time replaced by so-called "diffuser tiles," which are porous tiles, each acting as a lid to a cast-iron box, which is fitted with a separate air valve, in order that the air supply may be adjusted for the differences in porosity of individual tiles.

These diffusers may be arranged on the "ridge and furrow" system, being situated at the bottom of equally spaced furrows across the bottom of the tank at right angles to the direction of flow of the sewage. The ridges between eliminate any dead areas in which sewage might settle out, as any sludge which settles on a ridge and rolls to the bottom of a furrow is immediately swirled away by the air issuing from the diffuser.

A second arrangement, the "spiral flow" method, has the diffuser tiles situated along one side of a long narrow tank. The air issuing from the tiles causes a rotary movement to be given to the sewage, which, combined with the longitudinal flow down the tank, results in a corkscrew motion of the tank contents. This is often assisted by deflectors suitably placed at the surface of the tank.

Of the two methods, the spiral flow system results in a slight economy of air, using per gallon of sewage 0·8 cub. ft. against 1·1 cub. ft. for the ridge and furrow system.

BOLTON (SIMPLEX) SYSTEM. Mechanical means are used to provide the necessary aeration, a hopper-bottomed tank having in the centre a submerged tube, above which, and only slightly submerged in the sewage, is a power-driven revolving cone. The revolution of this at a high speed draws the sewage and sludge up the vertical pipe, and discharges it in a spray over the surface of the tank; the sewage thus continually passes down the sides and up the centre of the tank, oxygen being absorbed from the atmosphere during the transit.

SHEFFIELD (HAWORTH) SYSTEM. Paddle wheels are employed in this method to drive the sewage and sludge through a series of channels constructed in a zigzag manner, a large bulge being made at the end of each intercepting wall, causing a constriction in the channel, which results in a combined speeding up and rolling motion of the sewage. The channels are from 3 ft. to 5 ft. deep, and the sewage flows at about 1·5 ft. per second. The oxygen absorption takes place due to the continually changing surface of the sewage exposed.

ESSEN-RELLINGSHAUSEN SYSTEM. This method is a combination of the Sheffield and compressed-air principles. Long tanks, 10 ft. wide and 10 ft. deep, have paddles fitted along their longitudinal axes, power driven at about 7 r.p.m. Compressed air is blown through diffusers down one side of the tank, which are placed in such a manner that the injected air meets the paddles, thus causing the air to be kept in contact with the sewage for a longer period than in other methods; the air consumption is only 0·13 cub. ft.

Whatever the method employed for aeration, the aerated sewage, together with the activated sludge and any new sludge formed, passes into the sedimentation tanks, which follow the aeration units. A well-aerated sludge settles out in a few minutes, leaving a clear top liquor. Most of this sludge is returned to the incoming sewage to keep up a correct percentage, the effective proportion for good purification being, however, a much debated point.

Harris, of Glasgow, as a result of some interesting experiments in May, 1929, has obtained a "coefficient of interfacial contact," which is the product of the percentage of sludge and hours of contact. By regulating this factor to 30, a percentage purification of 65 per cent to 74 per cent on the oxygen absorbed basis was obtained. In most English works, an amount of sludge equivalent to 12 per cent on the whole tank contents is usually considered necessary.

The average power consumption per million gallons treated approximates to 20–25 b.h.p. hours.

CONTACT AERATORS. This is a method of pre-treatment of sewage before employing one of the biological processes outlined. Fresh sewage may be half treated, and such treatment results in considerable economies; one-half of the oxygen demand due to colloidal impurities is eliminated, with the result that subsequent treatment may be planned on half scale.

Contact aerators may be regarded as bacteria beds operating under water, and are also known as Emsher filters. The purification is obtained chiefly by fungi and, in consequence, a periodic cleaning away of the old, long filaments is necessary in order to make room for the new and more active growths.

Tanks 6–10 ft. deep are used, in which an air pipe swings like a pendulum; the bottom of the tank is curved parallel to the arc of the pendulum. The pipe is perforated with $\frac{1}{4}$-in. holes, 6 in. apart, and has an oscillation period of about four per minute.

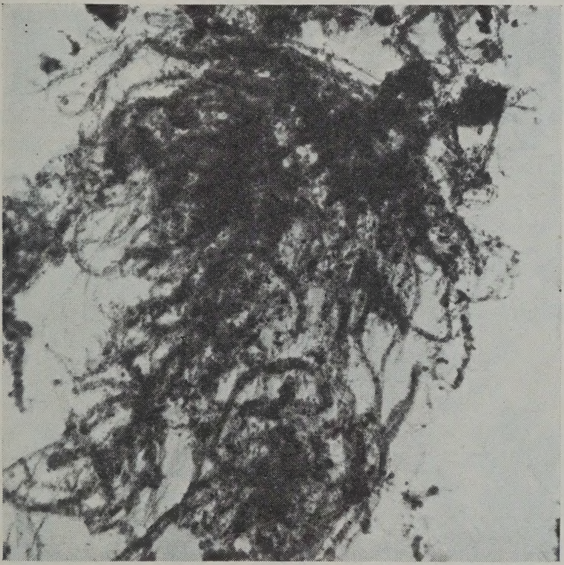


FIG. 205. ACTIVATED SLUDGE, SHOWING THE FILAMENT STRUCTURE OF THE AGGREGATES

The filaments are grown on boxes constructed of horizontal wooden laths, which are placed parallel to the swinging pipe, and so arranged that the sewage is lifted through the boxes, and descends between them.

Chemical Precipitation. The old settling tank, often much mismanaged, was found unsatisfactory when authorities in this country began to deal with sewage in a scientific manner, and chemical precipitants were amongst the first aids to purification to be investigated.

It is often a very difficult matter to find a suitable precipitant, and failures have been common, in addition to which the treatment of the sludge after precipitation was not easy, until the introduction of the filter press.

Chemical precipitants may act in one of two ways. The first method is to produce a voluminous precipitate, which carries down with it fine suspended matter, whilst the second throws out dissolved matter in the form of a precipitate. Lime is almost always used, though usually in combination with copperas, aluminoferric, basic sulphate, or sulphuric acid may be employed alone.

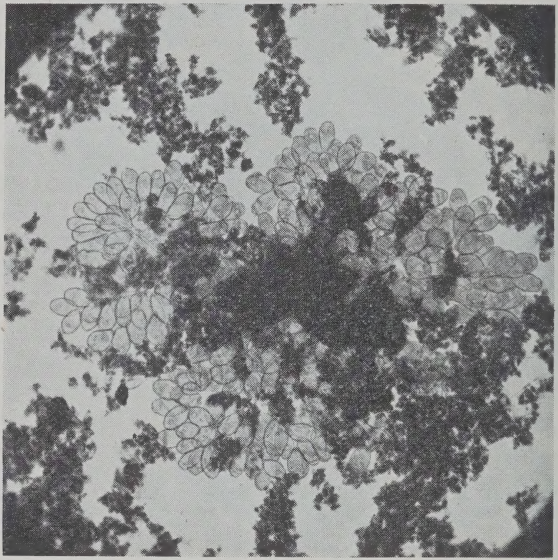


FIG. 206. ACTIVATED SLUDGE, SHOWING VERY LARGE COLONY OF *Vorticellae* ROUND SLUDGE PARTICLE

Aluminoferric is made up into blocks which may be added to the incoming sewage, being slowly dissolved by the flowing stream of liquid; milk of lime is usually run into the sewage afterwards. Lime may be used alone, the precipitated carbonate carrying down organic matter, but excess must be carefully avoided, as lime dissolves certain organic matters which are normally insoluble. Copperas may also be used with lime, the precipitated hydroxide carrying down the impurities. The iron also acts as an oxygen carrier.

The quantities found to be effective are, per 100,000 parts of sewage, 6 parts of lime, 1·5 parts of copperas; or 2 parts of lime and 1 part of aluminoferric. An excess over the quantities which give a good result has no beneficial action.

When sulphuric acid is used, an excess of about 10 parts per 100,000 may be considered sufficient to obtain good precipitation. Acid is employed only when grease is to be recovered from the sewage.

Sludge Disposal. This question has long been the bogey of sewage engineers, particularly in the case of sludge from activated sludge tanks. The American method is to treat by vacuum filters and rotary driers, obtaining a perfectly dry powder rich in nitrogen, which is sold as an organic manure. Centrifugal separation has also been used, reducing the bulk to one-fifth, but the residue is still a wet mass, and the method is expensive.

ARTIFICIAL DRYING. Normally, artificial drying is employed only when a saleable commodity for manure, or a solvent extracted grease, is required. Rotary dryers were the first to be used, and consist of a long, slightly inclined jacketed cylinder, which slowly rotates. The sludge is fed in at the top, where the hot air enters, and gradually works down at the bottom of the cylinder. The hot air, which is obtained from hand-stoked coke fires, traverses the interior of the dryer, returning through the jacket.

An 80 per cent sludge has been successfully reduced to a 15 per cent water content, but sludge of such high water content tends to stick to the sides, resulting in overheating and firing, or "balls" when imperfect drying ensures. In America rotary dryers are therefore often worked in combination with a vacuum filter.

A dryer which has not yet been adopted commercially for sludge drying, but is undergoing bulk trials at one large works, is a band dryer such as is used in the beet sugar industry; 100 tons per day may be treated, producing an almost dry powder from a wet mass. The machine consists of two chambers, the upper one providing a slow preliminary drying, the lower and hotter chamber completing the process, at the same time concentrating the sludge into a much thicker layer, by reason of the slower travel of the drying band. Hot air is the drying agent, worked on the counter-current principle.

Ordinary settling tank sludge contains about 90 per cent of water and is often treated by mixing with 1 per cent of milk of lime, following by filter pressing for 90 minutes which transforms it into a hard cake which still contains 50 per cent of water. The sludge cake may be air dried for some time, and burned in the boilers; it is of little use as a manure since the lime soaps make it slightly waterproof.

Sludge containing a fair amount of grease may be hot pressed with sulphuric acid. As an example, a sludge containing 85 per cent water may be reduced in 72 hours by alternate steaming and sludging, to a hard cake having a water content of 28 per cent, the grease in the original sludge (40 per cent of the total solids) being reduced to 15 per cent in the cake. The grease expressed may be sold and adds considerably to the revenue of many works.

Sludge Drying Beds are constructed of gravel, clinker, or crushed stone, 10 in. deep, with the coarse material at the bottom, and a superficial layer of fine asbestos sand. The sand is usually removed with the dried sludge and must be replaced after each application; it prevents the sludge draining into the bed. Ten inches of sludge may be spread on to such a bed, and this takes about three weeks to dry sufficiently for removal with a spade.

A bed will deal with perhaps a dozen lots of sludge in the year. In some cases, new lots of sludge are added to the old dry lots without removal, in which case the beds require to be four times as large. Planks or concrete walks are placed every 3 ft. or so in order to prevent the bed being powdered by workmen walking on them during the removal of the sludge cake.

Sludge Trenching. Trenches are cut into the land, in this method, 3 ft. wide and 2½ ft. deep, in which the sludge is placed. The trenches are set 6 ft. apart, and are served by a common trench. Sludge is run in every day until the trenches are full, after which a second set of trenches is cut between the original ones. After the second year, trenches are made at right angles to the first, thus ensuring a thorough mixing of the sludge with the soil. One large sewage works has effectively treated 80 tons of sludge per day, of 90 per cent water content, on 18 acres of land, over a large number of years.

The area of land required is, per head of population, weak sewage, 1·25 sq. yards; average sewage, 1·5 sq. yards; strong sewage containing trade waste, 1·75 sq. yards. When the grease content is high, however, the sludge is very difficult to dry. The addition of lime to the sludge speeds up the drying very considerably.

Sludge Digestion. This method is now widely used in the dewatering of sludges from all methods of treatment. Activated sludge, and humus sludge, which have hitherto presented a multitude of difficulties, have been successfully treated by the action of anaerobic organisms, and a reduction of 75 per cent of the original bulk obtained. The resulting sludge gives no difficulty in drying by air, whilst the untreated sludges are almost impossible to air dry in a reasonable time.

There are at present two methods in general use, the separate sludge digestion, and the Imhoff two-story tank process.

The former method is employed to deal with a sludge produced by some other process such as simple sedimentation or activated sludge, the object being to concentrate the sludge into much smaller bulk. The Imhoff process employs a top tank as a sedimentation chamber, the sludge settling down on to a cone outlet, and passing in a much concentrated form into the bottom chamber which acts as a digester, on the continuous flow system. The continuous flow keeps the temperature of the tank slightly higher than that of the atmosphere, thus accelerating the activity, whereas the separate type often requires the introduction of a heating device.

In both instances, during the decomposition of the sewage, acids are first formed upon starting up a tank, the *p*H figure dropping to about 5·0, with the evolution of large amounts of CO and hydrogen, concurrently with the production of acetic, lactic, and formic acids. This action is quickly followed by the formation of ammonia and other basic decomposition products, causing the sludge to become alkaline, whilst simultaneously the production of hydrogen falls, giving place to the evolution of methane and nitrogen. The gases are led off and in modern plant are utilized for heating, etc.

The factors which influence the decomposition in bulk are four. The introduction of fresh sludge must be carried out in such a way that there is thorough inoculation by the old active sludge, or acid fermentation will result, and the tank be put out of action. The ratio of tank capacity to the amount of sewage introduced at a uniform rate must be such that the retention period is about two months. The temperature may range between 42° F. and 77° F., the optimum temperature being different for each type of sewage. Finally, the *p*H value of the sludge should be from 7 to 7·6, but this factor automatically adjusts itself if the other three conditions are correct.

The cause of the sludge liquefaction is probably due to enzymes; both diastatic and proteolitic enzymes occur in large quantities, and when isolated from a digestion tank in a sterile condition produce the desired decomposition of the sludge. The inclusion or development of suitable bacteria, however, naturally accelerated the action considerably.

The methane produced may be commercially utilized, and at the Birmingham works, gas hoods have been installed built of concrete, measuring 10 ft. by 20 ft., and weighing about 8 tons each. They are domes, and fitted with draw-off pipes, several hoods being connected together and the pressure equalized. The gas obtained contains 67 per cent methane, 30 per cent carbon dioxide, and 3 per cent other gases; its thermal value is 625 B.Th.U.'s per cubic foot, as compared with normal town's gas which has a thermal value of 430. The estimated yield is 150,000,000 cub. ft. per annum, equivalent to 1,000 b.h.p. continuously day and night; calculating the value of the gas at 1s. 6d. per 1,000 cub. ft., this is equivalent to a revenue of £11,000 per annum. In September of 1927 a gas engine of 150 b.h.p. was installed to drive a 100-kW alternator on the gas produced.

Continuous Pressing. At the Milwaukee sewage works an installation of 24 Oliver vacuum filters is used. These filters consist of an outer cylindrical porous shell, which revolves on a horizontal axis, and contains an inner and impervious drum. The space between the two cylinders contains vacuum pipes and draw-off pipes.

The drum is immersed in the sewage rather less than half-way and slowly rotates. As the section submerged is under vacuum, a cake begins to build, continuing to form until the point of emergence is reached. At a later point of the rotation the cake is scraped off.

The activated sludge at Milwaukee is reduced from 90 per cent to 70 per cent water content, and is finally dried by means of a rotary dryer.

Hot Pressing. Districts such as Bradford, which, owing to the large number of wool-scouring firms in the area served, have a considerable amount of grease in the sludge, rely on hot pressing for their sludge disposal, as grease recovery is automatically effected. The sludge is precipitated by means of sulphuric acid, and contains from 85 per cent to 90 per cent of water. It is boiled in batches with steam and forced into the presses by means of compressed air. The pressing operation consists in alternately sludging and steaming, and lasts on an average about 60 hours. The final cake contains 38 per cent of water and 15 per cent grease, against 40 per cent of grease in the original sludge (calculated on dry solids), whilst the effluent is a mixture of grease and water. The grease is separated by gravity, purified with sulphuric acid, and sold, whilst the water is returned to the incoming sewage.

Solvent Extraction. A further method of extracting grease is by means of solvents, and is increasing in use, as the dried sludge is much more suitable as a manure, owing to the absence of grease, which is detrimental to manures, and also to the more complete extraction and recovery of the grease.

It is first necessary to dry the sludge artificially, by one of the methods already indicated. The dried sludge is extracted in 3-ton lots, by the solvent, which is often pre-heated. The grease-solvent mixture is distilled in a continuous still, to recover the solvent.

Sewage grease is dark brown or black in colour, with an unpleasant smell; it contains from 25 per cent to 30 per cent of free fatty acids, calculated as oleic.

Heavy axle greases have been made from it, but it is chiefly distilled at stearin works.

Houston's Standards for Effluents.

Total bacteria: Gelatine plate 20° C. . Less than 100,000 per c.c.
Agar plate 37° C. . Less than 100,000 per c.c.
Esch. coli . . . . . Less than 1,000 per c.c.
B. enteritidis sporogenes gas test in gelatine shake. None with 0·1 c.c.
Indol, neutral red broth test. Lactose bile broth test and litmus milk test. No result with 0·001 c.c.

Standard for Drinking Water. Esch. coli should be definitely absent in 100 c.c., and is very preferably absent in 500 c.c.

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CHAPTER XVII

FORMULAE FOR REAGENTS

1. **Botanical Stains.** General formulae—

1 grm. dyestuff in (*a*) 100 c.c. water;
(*b*) 100 c.c. 95 per cent alcohol;
or (*c*) 70 c.c. alcohol + 30 c.c. water.

2. **Aniline Water.**

A saturated solution of freshly distilled aniline in water. The aniline should be kept in a bottle, which is wrapped round with brown paper in order to exclude light; it will then keep longer. For use in general formulae, see No. 5 (Bacteriological Stains).

3. **Alcoholic Aniline.**

10 c.c. freshly distilled aniline;
90 c.c. 95 per cent alcohol.

For use in general formulae, see No. 5.

4. **Stock Dye-stuff Solutions.**

A saturated solution is prepared by adding an excess of the dye to a few cubic centimetres of alcohol in a test tube, heating on a water bath, cooling and filtering after standing 2 hours in cold water. See Nos. 6 and 8.

5. **Aniline Stains.** General formula—

25 c.c. alcoholic aniline, No. 2;
75 c.c. water (giving about 20 per cent alcohol);
1 grm. dyestuff.

6. **Aniline Water Stains.** General formula—

1 c.c. stock, No. 4;
10 c.c. aniline water.

Mix well, filter through wet filter paper into reagent bottle. Keeps for a few weeks only.

7. **Stains.** General formula—

0·1 grm. dye;
7 c.c. distilled water;
7 c.c. alcohol.

For use, mix 5 c.c. with 50 c.c. distilled water.

8. Stains. General formula—

1 c.c. stock (No. 4);
10 c.c. water.

9. Stains. General formula—

1 c.c. stock (No. 4);
10 c.c. water, 1 : 1 alcohol.

10. Ehrlich's Aniline Gentian Violet.

Make up according to formula No. 6.

11. Löffler's Methylene Blue.

3 c.c. stock (No. 4);
10 c.c. aqueous NaOH, 0·1 grm. per litre.

12. Mucillage Methylene Blue.

0·2 grm. methylene blue;
10 c.c. absolute alcohol;
40 c.c. glycerine.

13. Giemsa's Stain.

0·4 grm. Giemsa's powder;
25 c.c. glycerine;
75 c.c. pure methyl alcohol.

14. Carbol Fuchsine (Ziehl)—

1 grm. basic Fuchsin;
10 c.c. absolute alcohol;
100 c.c. 5 per cent phenol.

15. Gram's Stain.

0·1 grm. gentian violet;
7 c.c. absolute alcohol;
63 c.c. 1 per cent phenol.

For *Gram's iodine,* see No. 19.

16. McCrorie's Night Blue.

A. 1 grm. tannic acid;
20 c.c. water.
B. 1 grm. potash alum;
20 c.c. water.

Mix *A* and *B*, and to the mixture add—

C. 0·5 grm. Night Blue;
20 c.c. absolute alcohol.

17. Soudan Glycerine.

0·01 grm. dye;
5 c.c. alcohol; dissolve and add—
5 c.c. glycerine.

18. Cotton Blue.

0·05 grm. Cotton Blue;
50 c.c. lacto phenol (No. 79).

19. Meyer's Stain.

1 grm. Sudan III;
20 c.c. absolute (or 95 per cent) alcohol.

20. Dyestuffs Used as Stains.

Methylene Blue: Basic dye, soluble in water and alcohol. Spectrum: 667·8 and 609·3.

Gentian Violet (Pykotannin): A mixture of methyl violets. Basic colour, soluble in water and in alcohol. Spectrum: 587·0 and 535·0.

Acid Fuchsine (Acid magenta, Rubine): Acid dyestuff, soluble in water, insoluble in alcohol. Spectrum: 549·5 and 338·0.

Basic Fuchsine (Magenta): Basic colour, soluble in water and alcohol. Spectrum: 546·5 and 489·2.

Eosine: Soluble in water and alcohol. Spectrum: 516·3 and 483·5.

Bismark Brown (Vesuvine): Basic colour, soluble in water and alcohol.

Safranine: Basic colour, soluble in water and alcohol. Spectrum: 539·0 and 503·2.

Neutral Red: Azine dyestuff, indicator action, soluble in water and in alcohol.

See also: Fairbrother, "Chemistry and Application of Microscopic Stains." (*Industrial Chemist,* March, 1926.)
Lee, *The Microtomist's Vade Mecum.*
Rowe, *Colour Index* (Society of Dyers and Colourists).
British Dyestuff Corporation, Ltd., *Some notes on Micro Stains.*

21. Iodine-sulphuric Acid.

A. 3 grm. potassium iodide;
1 grm. iodine;
300 c.c. water.

B. 3 parts concentrated sulphuric acid;
3 parts glycerine;
1 part water.

22. Gram's Iodine.

1 grm. iodine;
2 grm. potassium iodide;
300 c.c. distilled water.

Shake occasionally for an hour or so until dissolved, and keep in a stoppered bottle.

23. Herzberg's Iodine-sulphuric Acid.

A. 1 grm. iodine;
5 grm. potassium iodide;
100 c.c. water.
B. Sulphuric acid, s.g. 1·45.

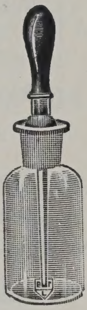

FIG. 207

24. Wallis's Mounting Iodine.

5 c.c. N/10 iodine;
100 c.c. water.

25. Wallis's Irrigation Iodine.

50 c.c. N/10 iodine;
50 c.c. water.

26. Herzberg's Zinc Chloride-iodine.

A. Zinc chloride, S.G. 2·00.
B. 30 grm. potassium iodide;
50 c.c. water.

Iodine added until excess is present after several days.
For use, decant 9 c.c. of *A* and add 1 c.c. of *B*.

27. Mercuric Chloride.

A saturated solution in 1 per cent acetic acid.

28. Osmic Acid.

One per cent aqueous solution, containing a trace of hydrogen peroxide.

29. Picric Acid. A saturated solution in either—

(*a*) water; (*b*) alcohol.

30. Picro-mercuric Chloride.

2·5 grm. mercuric chloride;
100 c.c. hot water.

When dissolved, add—

1 grm. picric acid.

Cool, and add—

10 c.c. 40 per cent formaldehyde (just before use).

31. Flemming's Fixative.

2·5 c.c. chromic acid, 1 per cent;
1·0 c.c. acetic acid, 1 per cent;
to 9·0 c.c. with water;
add 1·0 c.c. osmic acid, 1 per cent (immediately before use).

32. Chromic Acid.

1 c.c. acetic acid;
100 c.c. aqueous chromic acid, 1 per cent.

33. Potassium Bichromate.

2–5 per cent aqueous solution.

34. Alpha Naphthol.

10 per cent alcoholic solution.

35. Aniline Hydrochloride.

A saturated aqueous solution of the hydrochloride made slightly acid with hydrochloric acid.

36. Phloroglucinol.

A 1 per cent alcoholic solution. Should not be used if more than a month old.

37. Ferric Chloride.

A 1 per cent aqueous solution (N.B : Is almost always acid in reaction).

38. Alcoholic Ferric Chloride.
A 1 per cent solution.

39. Etherial Ferric Chloride.

A 1 per cent solution of dry ferric chloride in ether.

40. Tannin-ferric Chloride.

10 c.c. ferric chloride, 2 per cent;
30 c.c. saturated aqueous tannic acid.
Filter before use. Improves with age.

41. Muir's Mordant.

2 c.c. saturated mercuric chloride;
2 c.c. 20 per cent aqueous tannic acid;
5 c.c. saturated potash alum solution.

42. Millon's Reagent.

3 c.c. mercury;
27 c.c. concentrated nitric acid.
Dissolve cold, then add an equal volume of water.

43. Braemar's Reagent.

1 grm. sodium tungstate;
2 grm. sodium acetate, make up
to 10 c.c. with water.

44. Ruthenium Red.

1 c.c. of 10 per cent lead acetate is taken, and sufficient red is added to give a red coloration. The stain must be freshly prepared.

45. Grenacher's Borax-carmine.

5 grm. carmine;
8 grm. borax;
480 c.c. water. Heat to dissolve, cool; add—
480 c.c. water; stand several days, and filter.

46. Picro Carmine.

0·5 grm.	*A*. Carmine;
5 c.c.	Ammonia, sp. gr. 0·96;
100 c.c.	Water.
2·0 grm.	*B*. Picric acid;
100 c.c.	Alcohol.

Mix *A* and *B* for use.

47. Corallin Soda.

A 30 per cent solution of sodium carbonate is added to an alcoholic corallin solution until the liquid is bright pink. The stain must be freshly prepared.

48. Alkanna.

20 grm. alkanna root;
100 c.c. alcohol, 90 per cent.

Macerate one week, boil 10 min., and cool. For use, dilute with an equal quantity of water.

49. Brazelein.

Mordant:	1 grm. iron alum;
	100 c.c. 75 per cent alcohol.
Stain:	1 grm. brazalian crystals;
	100 c.c. 75 per cent alcohol.

50. Delafield's Haematoxylin.

200 c.c. saturated alum. Add very slowly—
2 grm. haematoxylin;
12 c.c. absolute alcohol.

Expose to light and air for one week, filter, and add—

50 c.c. pure glycerine;
50 c.c. methyl alcohol.

Stand in the sun until the colour develops (this may take two months in winter). Filter to remove precipitated ammonium alum, and keep in a tightly-corked bottle. The stain is stable for many years.

51. Reaction of Nutrient Media.

The growth of micro-organisms on nutrient media is dependent to a greater or lesser extent on the reaction of the medium. For this reason the acidity is adjusted to an exact value, which is usually expressed on "Fuller's scale" by means of a number preceded by either a + or a – sign, e.g. + 15. The number indicates the departure from neutrality in cubic centimetres of normal hydrochloric acid, acidity being represented by + and alkalinity by –, phenolphthalein being the indicator. (The point of neutrality to litmus is thus + 25.) It is important to note that the pH value is not the same when other acids are substituted for HCl.

Five c.c. of the medium are diluted to 50 c.c. with water in an evaporating dish, boiled 3 min. to drive off CO_2 and titrated to phenolphthalein with N/20 HCl. Three titrations should be carried out and averaged. The whole of the medium is now treated with the correct quantity of acid required to bring it to neutrality, a little is checked by titration, and finally the requisite amount of acid is added to give the Fuller's scale reading desired. The optimum reactions for various classes of organisms are—

Animal pathogens: + 5.
Water bacteria: + 10 to 15.
Putrefying bacteria: + 10 to 15.
Fungi: + 15 to 25 or more.

A value of + 10 is a very generally useful one. In descriptions of cultures the reaction should always be stated. For many purposes it is sufficient to make the media alkaline to litmus, but acid to phenolphthalein, i.e. between + 10 and + 25.

52. Nutrient Broth.

12 grm. peptone;
2 grm. sodium chloride;
50 c.c. 10 per cent Lemco flesh extract solution;
950 c.c. water.

Adjust reaction, filter through filter paper, run into 100 c.c. conical flasks (for storage), plug with cotton wool, and sterilize for half an hour in steam on each of three successive days.

53. McConkey and Hill's Lactose Bile Broth.

0·5 grm. sodium taurocholate;
0·5 grm. glucose;
2 grm. peptone;
100 c.c. water.

Heat to dissolve, add neutral litmus to give a decided red colour, and sterilize.

54. Flesh Water.

500 grm. flesh (no fat);
1,000 c.c. water.

Steep 24 hours cold, press, boil the extract half an hour, filter through muslin, and add (per litre)—

5 grm. sodium chloride;
10 grm. peptone.

Adjust reaction, filter, and sterilize.

55. Maltose Peptone Broth.

4 grm. maltose;
1 grm. peptone;
2 c.c. N/2 HCl;
100 c.c. water.

Other sugars may be substituted for maltose.

56. Gelatine Medium.

A. 12 grm. peptone;
2 grm. NaCl;
80 c.c. 10 per cent Lemco flesh extract solution;
320 c.c. water.

Heat in a plugged flask on the water bath until solution is complete.

B. 100 grm. gelatine (i.e. gives finally 10 per cent);
600 c.c. water.

Steep overnight in a plugged flask, and warm up next morning until dissolved; or the mixture may be heated on the water bath directly until dissolved, though the resultant jelly does not set so solid.

Pour *A* into *B*, and make alkaline to litmus, but acid to phenolphthalein; or adjust the reaction exactly. Steam sterilize for

20 min., cool to 40–50° C., add the beaten white of an egg, shake up well, and again sterilize. Filter through a Buchner funnel and sterile cotton wool to remove the coagulated egg albumen, run into 100 c.c. conical flasks for storage, and plug. Sterilize for half an hour in steam on each of three successive days. It is advisable in very accurate work to plate out some of the medium and incubate at 20° C. for some days to make sure that the sterilization is complete.

A further and often very convenient method of clarification is to filter the medium through filter paper in the steam sterilizer.

57. Sugar Gelatine Media.

Add 10 grm. of the sugar to *A* in formula No. 56.

58. Plum Gelatine.

100 grm. dried plums;
100 c.c. water.

Make up to 200 c.c., and add 20 grm. gelatine.

58. Potato Gelatine.

100 grm. grated potato;
300 c.c. water.

Stand overnight, strain off 300 c.c., heat in water bath for an hour, filter, add 4 per cent glycerine, and sterilize.

60. Nutrient Agar.

A. 12 grm. peptone;
2 grm. sodium chloride;
80 c.c. 10 per cent Lemco flesh extract solution;
320 c.c. of water.
B. 15 grm. agar (giving finally 1·5 per cent);
600 c.c. water.

Proceed exactly as with No. 52, but omit the clarification with egg albumen. The melting point is 95° C. and, therefore, after melting (by immersion in boiling water), care must be taken to cool it down almost to its setting point—about 40° C.—before inoculating for agar plates. Bacteria, except thermophilic and spore-forming ones, are, of course, easily killed at a temperature much above 42° C. Agar plates should be incubated upside down (at 37° C.), in order that the steaming of the top dish may not have, as a consequence, drops of water on the medium, which would cause some colonies to "run."

61. McConkey's Lactose Bile Agar.

10 grm. peptone;
2·5 grm. sodium taurocholate;
7·5 grm. agar (i.e. 1·5 per cent);
500 c.c. water.

Heat in the sterilizer until solution is complete, cool, mix with the white of an egg to clarify (see No. 52), filter, add the lactose, and sterilize.

62. Maltose Peptone Agar.

4 grm. maltose;
1 grm. peptone;
2 c.c. N/2 HCl;
1·5 grm. agar;
100 c.c. water.

63. Sugar Agar.

Add 10 grm. sugar to *A* in No. 60.

64. Blakeslee-Reddish Wort Agar.

100 grm. Difco dried malt extract;
900 c.c. distilled water.

Make to 8 degrees Kaiser (saccharimeter) with about 100 c.c. distilled water; make to + 1·5 reaction; add 15 grm. agar; autoclave, 15 lb., 15 min.; filter; tube; and sterilize 15 lb., 10 min.

65. Fruit Juices.

200 grm. dried fruit;
1,000 c.c. tartaric acid solution, 4 grm. per litre.

Stand 24 hours, press, filter, and sterilize.

66. Dox-Czapek Agar.

2 grm. sodium nitrite;
1 grm. dipotassium phosphate;
0·5 grm. magnesium sulphate;
0·5 grm. potassium chloride;
0·01 grm. ferrous sulphate;
30 grm. sucrose;
15 grm. agar;
1,000 c.c. water.

Proceed as usual.

67. Yeast Water.

250 grm. pressed yeast;
1,000 c.c. water.

Sterilize Pasteur, filter, and sterilize Tyndall; dilute with sterile water until reddish amber in colour.

68. Liquid for Preserving Yeasts.

100 c.c. 10 per cent cane-sugar;
0·1 grm. sulphuric acid.

Sterilize. Preserve the yeasts in Hansen flasks, the outlets being plugged with cotton wool, and sealed with sealing wax.

69. Yeast Sugar Media.

Yeast water (No. 67) containing 10 per cent sugar. Solid media may be prepared by adding 1·5 per cent agar.

FIG. 208

70. Pasteur's Fluid.

75 grm. pressed yeast;
1,000 c.c. water.

Boil a quarter of an hour, filter, make up to volume, sterilize, and add 5–10 per cent of cane sugar. Tube.

71. Bread Mash.

Dry crumbled bread is made into a paste with water, and sterilized.

72. Rice Mash.

10 grm. rice meal;
15 c.c. milk.

Maltose peptone broth.

73. Dubos's Medium. For cellulose decomposing organisms—

0·5 grm. sodium nitrate;
1·0 grm. dipotassium phosphate;
0·5 grm. magnesium sulphate;
0·5 grm. potassium chloride;
0·01 grm. ferrous sulphate;
1,000 c.c. water.

Add sterile filter paper strips.

74. Omelianski's Medium.

1·0 grm. dipotassium phosphate;
0·5 grm. magnesium sulphate;
0·5 grm. ammonium phosphate (or sulphate).

Trace sodium chloride; some chalk.

1,000 c.c. water.

75. Winogradski's Silica Jelly.

A. 0·4 grm. ammonium sulphate;
0·05 grm. magnesium sulphate.
Trace calcium chloride.
50 c.c. water.
B. 0·1 grm. potassium phosphate;
0·6 grm. sodium carbonate;
50 c.c. water.

Sterilize *A* and *B* separately, cool, mix, and add in small quantities to a 3–4 per cent solution of silicic acid until a good jelly is obtained.

76. Glycerine Jelly. Some gelatine is steeped overnight in water, and next morning, after pouring off the unabsorbed water, 2 oz. of the jelly and ½ oz. of glycerine are placed in an evaporating basin. The mixture is evaporated down on the water bath, an inverted clock glass being placed above it, ½ in. away, in order to prevent dust getting in. When the desired consistency is reached (which is tested by putting a drop on a slide, "mounting" it with a cover circle, cooling under the cold water tap for half a minute, and wiping fairly roughly with a piece of cloth), the gelatine solution is cleared by filtration through a hot water funnel; or the filtration may be carried out very conveniently inside the steam sterilizer; 1·0 grm. phenol is now added, and the reagent bottle filled. The mountant requires melting in warm water before use. Most of the glycerine jelly mountants on sale are too thin.

77. Canada Balsam. This mountant is usually dissolved in xylol, though some prefer chloroform. When an especially durable preparation is required, turpentine is to be preferred to either; but xylol is very serviceable, with the one disadvantage that during many years it slowly makes tissues so transparent that some detail is lost. If it is preferred to make the mountant in place of buying its solution in xylol, precaution should be taken to have the resin perfectly dry by heating it in an oven until it is brittle on cooling before making up the solution. An acid sample of resin should be rejected. Canada balsam is probably the most permanent mountant, but as it has almost the same refractive index as cellulose it is more suitable for stained tissues or for polarized light examination than for unstained.

78. Calberla's Fluid, for preserving fungi.

10 c.c. water;
10 c.c. absolute alcohol;
10 c.c. glycerine.

79. Lacto Phenol.

10 grm. phenol;
10 c.c. lactic acid;
20 c.c. glycerine;
10 c.c. water.

80. Glycerine Tannic Acid.

10 grm. tannic acid;
100 grm. glycerine.

A waxy solid, m.p. about blood-heat, useful for mounting solid bodies, e.g. crystals, which are to be examined in air.

81. Liquid for Cleaning Slides, etc

0·5 c.c. ammonia, S.G. 0·880;
50 c.c. methylated spirit;
25 c.c. water.

Add rouge to a thin cream.

82. Cleaning Liquid for Canada balsam slides.

60 grm. potassium bichromate;
60 c.c. sulphuric acid;
100 c.c. water.

Boil the slides for half an hour.

83. Ringing Varnish.

Shellac dissolved in naphtha to a fairly thick paste.

84. Elastic Cement. India-rubber dissolved in petroleum ether is mixed with thick old gold size and shellac naphtha, and filtered warm through fine muslin. It is used for mounting cells for dry or wet work.

A. 4 oz. best orange shellac;
300 c.c. 90 per cent alcohol.
B. 60 c.c. saturated solution of pure rubber in petroleum ether.
C. 120 c.c. old gold size.

Add *B* and *C* to *A*.

85. Finishing Varnish.

75 grm. gum dammar;
25 grm. gum mastic;
150 c.c. benzol.

Filter into a mortar; add the pigment required, with thorough grinding, a small amount at a time. Then evaporate down in a watch glass. For use, take up with a brush dipped in benzol. The following pigments are suitable: zinc oxide, carmine, lampblack, ultramarine, verdigris, lead chromate, etc.

86. Gold Size.

25 grm. powdered white lead;
1 grm. yellow ochre.

Boiled linseed oil and red lead are boiled together for three hours and allowed to settle. Pour off the clear liquid, and again boil, adding the pigment mixture gradually until the required consistency is obtained.

87. Copper Glycerine.

16 grm. copper sulphate;
10 grm. glycerine;
150 c.c. water.

Add to this solution, 20 per cent NaOH until the precipitate at first formed just re-dissolves. A slight excess of NaOH should be present, or cuprous oxide is precipitated on boiling.

88. Ammoniacal Nickel Oxide. Nickel hydroxide is precipitated, boiled, filtered, well washed with faintly acid (HAc) water by decantation, drained at the pump, placed in concentrated ammonia in a stock bottle, and allowed to stand for a few days before decanting off 20–30 c.c. into the reagent bottle. Fill up the stock bottle with ammonia, and proceed thus until all the nickel hydroxide is used up.

89. Chloral Hydrate.

5 grm. chloral hydrate;
2 c.c. water.

90. Mayer's Albumen.

50 c.c. white of an egg;
50 grm. glycerine;
1 grm. sodium salicylate.

Shake well, and filter. The mixture does not keep at all well.

91. Oxygen Absorption Liquid.

2·5 grm. dry pyrogallol;
100 c.c. 10 per cent KOH solution.

92. **Acid Alcohol.**

2 drops HCl;
25 c.c. alcohol.

93. **Developer for Plates** (dish development only).

94. **Developer for Bromide Paper.** (See below.)

95. **Developer for Gaslight Paper.** (See below.)

		93	94	95
A.	Metol	1·25 grm.	2·0 grm.	2·0 grm.
	Hydroquinone	3·75	8·0	6·0
B.	Sodium sulphite crystals	30	15	10
	Water	400 c.c.	400 c.c.	400 c.c.
C.	Sodium carbonate crystals	67	80	81
	Sodium sulphite crystals	120	60	40
	Potassium iodide 10%	15 c.c.	6 c.c.	9 c.c.
	Water	400 c.c.	400 c.c.	400 c.c.

A is dissolved cold in *B*, the solution added to *C*, and the whole made up to 1 litre.

96. **Glycin Developer** for tank development.

9 grm. glycin;
27 grm. sodium sulphite crystals;
46 grm. dry potassium carbonate;
to 1,000 c.c. with water.

For use, dilute 100 c.c. with 500 c.c. of water.

97. **Hypo.**

10 per cent solution of sodium thiosulphate crystals.

98. **Acid Hypo.**

200 grm. sodium thiosulphate crystals;
25 grm. potassium metabisulphite;
to 1,000 c.c. with water.

99. **Potassium Bismuth Iodide.** A concentrated solution of potassium iodide is added to a solution of basic bismuth nitrate in dilute hydrochloric acid until there is no precipitate on dilution.

100. **Potassium Mercuric Thiocyanate.** One c.c. of concentrated nitric acid is added to 100 c.c. of a saturated solution of mercuric nitrate. Filter, add 5 per cent KCNS until no further precipitate is formed. Filter, wash the precipitate until the washings give no further reaction with $FeCl_3$ or KI. Add the $Hg(CNS)_2$ to a 5 per cent solution of KCNS, which should be in excess, and stand for a few days. Filter, and add 5 c.c. KCNS solution per 100 c.c. of the solution, and evaporate down on a water bath. Allow to stand, when the double salt crystallizes in needles. Recrystallize from water. The solid keeps well.

101. **Etching Liquor** (ferrous alloys).

2 c.c. concentrated nitric acid;
100 c.c. alcohol.

102. **Etching Liquor** (ferrous alloys).

4 grm. picric acid;
100 c.c. alcohol.

103. **Etching Liquor** (ferrous alloys).

2 grm. picric acid;
100 c.c. 25 per cent aqueous NaOH.

104. **Etching Liquor** (copper alloys).

25 grm. chromic acid;
15 grm. sodium sulphate;
100 grm. water.

105. **Etching Liquor** (copper alloys).

10 c.c. ammonia.;
1 c.c. hydrogen peroxide, 12 vols.

106. **Etching Liquor** (aluminium alloys).

10 grm. NaOH;
100 c.c. water.

107. **Etching Liquor** (tin and lead alloys).

5 grm. silver nitrate;
100 c.c. water.

The silver deposit is rubbed off with the finger whilst washing the specimen under the cold water tap.

108. Beijerinck's Medium.

0·5 grm. mono-potassium phosphate;
0·5 grm. ammonium chlorate;
100 grm. gelatine;
1,000 c.c. water.

Wash gelatine well with distilled water to dissolve out soluble constituents. For some yeasts (*S. fragrans, S. Kefir, S. apiculatus*) replace NH_4Cl by 5–10 grm. peptone.

109. Peptone Water.

0·5 grm. sodium chloride;
1·0 grm. peptone;
0·2 grm. potassium nitrate;
1,000 c.c. water.

110. Ink for Writing on Photographs.

10 KI;
30 water;
1 iodine;
1 gum arabic.

Gives white lines on dark background.

111. Gibson's Medium.

60 c.c. alcohol;
30 c.c. water;
30 c.c. glycerine;
2 c.c. acetic acid 15 : 85;
0·15 grm. mercuric chloride.

112. Stevenson's Medium.

Mercuric chloride and potassium iodide, both added to water to saturation.

113. Eycleshymer's Medium.

10 c.c. cedar-wood oil;
10 c.c. bergamot oil;
10 grm. phenol.

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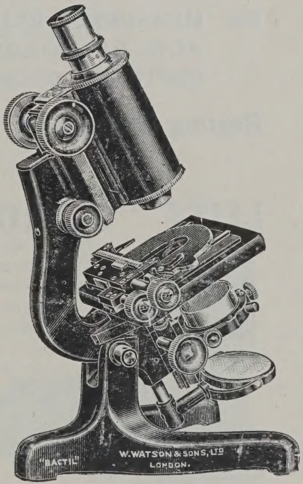

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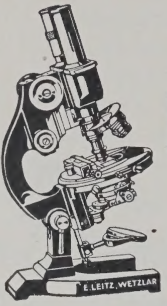

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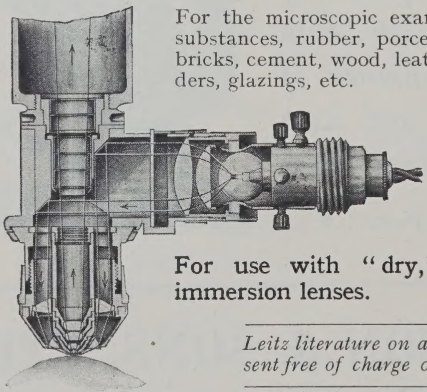

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